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PHYSIOLOGY OF POLLEN

B. M. Johri and I. K. Vasil

SEEDLING MORPHOLOGY IN APPLIED GENETICS AND PLANT BREEDING

Gordon Haskell

DIFFERENTIAL GROWTH-INHIBITORS PRODUCED BY PLANTS

Solomon Garb

FUTURE CONTENTS
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PHYSIOLOGY OF POLLEN*

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STORAGE

INTRODUCTION

Horticulturists and plant breeders have long been interested in crossing varieties, species and even genera to produce new and improved types of plants better suited to human requirements. However, many of these attempts have failed, due to the barriers to crossability, e.g., flowering of the selected parents at different times or at distant places, failure of the pollen grains to germinate on the stigma, bursting of pollen tubes in the style, failure of tubes to grow through the style, and slow growth of the tubes so that they do not reach the ovules before abscission of the flower. Occasionally the pollen tubes no doubt enter the embryo sac, but sterility results either from failure of the male gamete to fuse with the egg nucleus or from subsequent failure or arrested development of the embryo and the endosperm (see Blakeslee, 1945; Maheshwari, 1950).

Nowadays it is possible to store viable pollen; therefore the difference in the time of flowering of the parents does not hold up breeding programmes. The ease with which stored pollen can be transported long distances without loss of viability has partly solved the problem of the parents occurring in different regions. "The maintenance of pollen viability in a light weight container, and in an otherwise uncontrolled environment is of particular advantage in the long-distance shipment of pollens. Such shipments today require costly packaging and special precautions for maintaining viability enroute. Loblolly pine (*Pinus taeda*) pollen was shipped to New Zealand and return and to Brazil and return, unopened. Each sample, packed in "Starfoam," weighed slightly over one ounce when shipped, and each had lain on a laboratory shelf for nearly two months before shipment. Both packages, and their enclosed samples, returned in excellent condition having been enroute 26 and 29 days respectively" (King, 1959). Moreover, transport of pollen by air, land or sea is not controlled by plant protection and quarantine regulations.

The earliest reference on the handling and storage of pollen concerns the date palm. Zirkle (1935) states that records of certain business contracts of the Hammurabi Period indicate that male inflorescences of the date palm were an important commodity of commerce as early as 2000 B.C. However, we have no information about the methods of storage, but it is unlikely that the stored pollen retained viability in

the excessively low humidity and high temperature prevalent in those areas (Iran, Iraq, etc.).

Popenoe (1913) points out that "if date pollen is kept dry it preserved its value for a long time, and in some date-growing communities it is the custom to have a small supply from each year to the next ... A pollination made in 1912 at the Mecca Experiment Station with pollen seven years old, sent from the Temple Garden, was entirely successful." Some other reports (see Nebel and Ruttle, 1937) also indicate that pollen is capable of bringing about fertilization even after long periods.

Stout (1924), on the other hand, writes: "It remains to be shown whether date pollen can ever be kept viable from one season until the next season of bloom. The evidence at hand rather decidedly indicates that it is not and perhaps cannot be thus kept." In this connection attention may be drawn to the conflicting reports of Albert (1930) and Crawford (1937). Whereas the former was able to obtain a moderate fruit set in the following season after using the pollen of the previous year stored at room temperature, Crawford (1937) failed to get any such result with similar pollen.

The longevity of stored pollen has been investigated mainly in relation to relative humidity, temperature, effect of diluents and nutrient media (see Holman and Brubaker, 1926; Nebel and Ruttle, 1937; Visser, 1955; Vasil, 1958a, 1961; Johri and Vasil, 1960). These and some other related topics will now be considered.

EFFECT OF HUMIDITY AND TEMPERATURE

A systematic study on the storage of pollen was initiated towards the end of the nineteenth century when Mangin (1886), Rittinghaus (1886) and Molisch (1893) investigated the longevity of pollen of more than 80 species stored under air-dry conditions (exposed to room atmosphere). Pollen of tomato survives for four days under laboratory conditions (?) and gives normal fruits; older pollen causes reduced fruit-size (Gorobec, 1958). The pollen of several species remains viable for a longer period at lower temperatures than at higher (see Goff, 1901; Sandsten, 1909; Roemer, 1915). Both Sandsten (1909) and Roemer (1915) observed a longer viability under dry conditions. In 1922 Knowlton stored the pollen of *Antirrhinum* at five temperatures and concluded that the lower the temperature the longer the viability.

Pfundt (1910) investigated the effect of 0, 30, 60, and 90 per cent

relative humidity (R.H.) on the viability of the pollen of 140 species at 17°–22°C. From his observations it is evident that the maximum longevity was obtained at low relative humidities (0–30 per cent). His results were later confirmed by Holman and Brubaker (1926) who tested the viability of stored pollen of another 52 species at 17° –22°C, and at 0, 27, 63 and 92 per cent R.H. They concluded that "storage at low humidities triples on the average the longevity of those pollens which it affects." These authors have also reviewed the pertinent literature on this subject and have compiled data on the pollen longevity of 231 species belonging to 175 genera and 23 families.

Horsford (1918) reported a longevity of about one year in lily, and Manaresi (1924) observed a similar behaviour in apple, pear, grape and plum. Since then the pollen of numerous species has been reported to retain longevity for one year or even longer when stored at 0°–10°C and 10–50 per cent R.H. In this connection attention is called to the works of Nebel and Ruttle (1937), King and Hesse (1938) and Nebel (1939) on the pollen of several fruit trees; Pfeiffer (1936, 1938, 1944) on *Amaryllis, Cinchona* and *Lilium*; Gollmick (1942) and Olmo (1942) on *Vitis;* and Stone et al. (1943) on *Pistacia.*

In contrast to the relatively long viability in the above plants, the pollen of the Gramineae is extremely short-lived and the range of humidity (0–40 per cent), which is so favourable to most other pollens, is decidedly harmful. As a rule, the pollen of grasses remains viable only for a few days, sometimes one to three weeks, when stored at 0°–10°C and 80–100 per cent R.H. This is evident from the work of Pfundt (1910) and Andronescu (1915) on *Zea mays* and several other grasses; Firbas (1922) on rye and wheat; Anthony and Harlan (1920) and Pope (1939) on barley; Knowlton (1922), Sartoris (1942), Jones and Newell (1948), Bergh (1952) and Liefstingh (1953) on maize; Sartoris (1942) on sugarcane; and Jones and Newell (1948) on buffalo-grass (*Buchloë dactyloides*). This short life-span may be due to desiccation during storage.

Pfundt (1910) reports that the pollen of *Prunus padus* remains viable for 15 days at 90 per cent, 22 days at 60 per cent and 181 days at 30 or 0 per cent R.H. Similarly, the pollen of *P. avium* retains viability for 102 days at 30 per cent R.H., 126 days at 0 per cent, but only 28 days when stored at laboratory temperature and uncontrolled humidity. According to Holman and Brubaker (1926), in none of their

samples did the longevity of air-dry pollen exceed that of those stored over lower humidities. On an average, 27.2 per cent R.H. appeared to be slightly better than 0.005 per cent and much better than 94.2 and 63 per cent. The maximum longevity of 336 days in *Typha latifolia* was obtained when the pollen was stored at 17°–22°C over 0 per cent R.H. On the other hand, Knowlton (1922) mentions that the maximum longevity of maize is obtained at 50–80 per cent R.H. He concluded that desiccation appears to be one of the important factors that causes early death of pollen during storage. Nebel and Ruttle (1937) also state that "for the pollen of the species and varieties of *Prunus, Pyrus* and *Vitis* tested, a humidity of 50 per cent is possibly the optimum, the life curves becoming increasingly shorter and steeper as humidity is further increased and becoming shorter as humidity is further decreased." Again, according to Pfeiffer (1936), there is an intimate correlation between the longevity of pollen and moisture content of the air, with maxima and minima of different pollens at different humidities.

Visser (1955) points out that the longevity of pollen is, in general, negatively correlated with the relative humidity required for optimal storage. For instance, it is only at high humidity (80–100 per cent R.H.) that the pollen of the Gramineae retains its viability; and that, too, for only a short period. Daniel (1955) reports that the pollen of *Zea mays* remains viable for ten days at 7°C and 50–70 per cent R.H. Lower temperatures (–5° and –20°C) and less than 50 per cent humidity was detrimental. But the opposite holds true for the pollen of *Prunus* and *Pyrus* species which have a far greater viability at low humidities (30–50 per cent R.H.)

During the last seven years, investigations carried out at the University of Delhi indicate that the pollen of certain crop plants—*Arachis hypogaea, Brassica nigra, Pennisetum typhoideum, Solanum melongena* and *S. tuberosum*—shows maximum viability at 0 or 31–40 per cent R.H. Sometimes, especially at low temperatures, 50–60 per cent R.H. is also quite satisfactory (Vasil, 1958a, 1961).

Whereas over low humidities (0-40 per cent R.H.) the stored pollen remains viable for long periods, at high humidities (60 per cent R.H. and above) it is often attacked by bacteria and fungi which render the samples unfit for further use (Vasil, 1958a). Nebel and Ruttle (1937) also noticed the growth of mould in their pollen samples stored over 100 per cent R.H.

There is some recovery of the germinating capacity of *Cinchona* pollen upon transfer to favourable conditions of temperature and humidity after very dry conditions at room temperature (Pfeiffer, 1944; see also Nebel and Ruttle, 1937; Pfeiffer, 1955).

When the relative humidity is allowed to fluctuate frequently during storage, viability is lost quickly (Bullock and Snyder, 1946). It appears that pollen cannot withstand extreme variations in its environment.

As early as 1901, Goff (see also Sandstein, 1909) stated that in several fruit plants the longevity is not impaired after a short exposure to sub-zero temperatures. Griggs et al. (1953) have also shown that pollen can be stored for long periods in cotton-plugged vials in a home freezer (temperature close to 0°F) without any control of humidity. Under such conditions, the viability of hand-collected almond pollen was retained for 801 days, while bee-collected pollen remained viable up to 1130 days. Pollen of *Poncirus trifoliata* retained viability for 61 days over calcium chloride at 4°C (Soost and Cameron, 1954). Pollination with 36-day old stored pollen yielded fruits and viable seeds and the latter produced normal, healthy seedlings. In the same plant, Sawano (1954) reported a viability of 110 days over calcium chloride but nothing is known about the temperature at which pollen was stored. Pollen of eight species of *Nicotiana* stored at –5°C and 50 per cent R.H. for one year germinated almost as well as fresh pollen (Daniel, 1955). A temperature of 7°C with 18 per cent R.H. had a slightly adverse effect, while a humidity of 100 per cent proved definitely harmful. According to Knowlton (1922), the pollen of *Antirrhinum* remained viable longest at –18° to –30°C, and no reduction in its viability was noticed even when exposed for half an hour to a temperature of –190°C (liquid oxygen). The pollen of *Lilium* and *Amaryllis* (Pfeiffer, 1936, 1938), date (Crawford, 1937), grape (Olmo, 1942) and many other fruit plants (Antles, 1951; Griggs et al., 1953; Visser, 1955) can be stored for long periods at deep-freeze temperatures (ca –20°C) without being adversely affected. With similar conditions of storage, Ushirozawa and Shibukawa (1951) noticed a slight germination in the pollen of many fruit species even after nine years which is the longest viability so far recorded.

The pollen of apple and pear can be satisfactorily stored over dry ice (solid carbon dioxide) at –55° to –60°C (see Griggs et al., 1950; Antles, 1951). Bredemann et al. (1947) stored the pollen of *Lupinus* at –180°C (liquid air), and Visser (1951, 1955) stored the pollen of

apple, pear and tomato at –190°C. Pollen grains of apple stored for nine months in small, closed, glass vessels at –15°C showed 95 per cent germination which was as good as in fresh samples (Tupý, 1960). Visser (1955) reports that apple pollen, after being stored at –190°C, "germinated equally well and induced as good a fruit set after two years . . . as freshly collected pollen. Evidently the time 'stands still' for pollen when stored at –190°C." Bredemann et al. concluded that the viability of the pollen of *Lupinus* would remain unaffected for millions of years if stored over liquid air. Visser (1955) also points out that pollen stored at such low temperatures "can be given 'eternal life'," provided that it is resistant. However, contrary to general expectation, the pollen of maize shows a sharp decline in viabilty when stored at deep-freeze temperatures (Knowlton, 1922; Liefstingh, 1953).

INFLUENCE OF GASES AND PRESSURE

Besides temperature and humidity, certain other atmospheric conditions also affect the viability of pollen. According to Kellerman (1915), the pollen of *Citrus* retained a high percentage of germination when pre-dried and shipped under vacuum. In *Lilium* (Pfeiffer, 1936), apple and pear (Visser, 1955), reduced air pressure prolongs the viability. On the contrary the pollen grains of barley (Anthony and Harlan, 1920), *Antirrhinum* (Knowlton, 1922), sugarcane (Sartoris, 1942) and *Cinchona* (Pfeiffer, 1944) remain viable longer at normal than at reduced air pressure. High concentrations of carbon dioxide (this is automatically achieved when the pollens are stored over dry ice) also increase longevity (Knowlton, 1922; Antles, 1951), whereas storage in pure oxygen is less favourable (Knowlton, 1922). Pollen of *Pinus taeda* remains viable for 99 days when sealed in nitrogen gas following freeze-dry treatment and stored in an otherwise uncontrolled environment (King, 1959). Normal and fresh pollen of *Citrus* shows only 50 per cent germination. It loses viability more quickly in light than in dark. Longevity can, however, be increased by storing at high carbon dioxide concentrations (Resnik, 1958).

EFFECTS OF DILUENTS

The effects of various diluents (finely powdered dry materials mixed with the pollen) on the storage and viability of pollen has been studied by Pfeiffer (1948), Overley and Bullock (1947) and others. In re-

cent years, in order to minimise the deterioration, particularly due to desiccation, it has become usual practice (Antles, 1951) to use diluents during storage and transport of pollen. The useful effect of the diluent may be due to its regulating capacity on moisture and air around the pollen grains (Bullock and Overley, 1949).

There are over 30 different substances which have been tried as diluents; the more commonly used are lycopodium powder, powdered egg albumen, casein and talc (see Overley and Bullock, 1947; Pfeiffer, 1948; Bullock and Overley, 1949; Bullock and Snyder, 1946; Antles, 1951). The diluents can be used effectively only on dry and powdery pollen, since with sticky pollens they form large clumps and increase the possibility of their bursting. In high humidity (over 55 per cent) some of the diluents (eg., egg albumen) absorb moisture and become sticky, resulting in profuse bursting.

Diluents which adhere to the pollen reduce their viability and cause a significant decrease in fruit set (Bullock and Snyder, 1946). Antles (1951) states that when the electrostatic charge of the diluent material was opposite to that of the pollen grains, the diluting material gathered around the pollen grains, sometimes causing them to group. This resulted in less possibility for contact with the pistil of the flower during artificial pollination.

It may be of interest that at present diluents are widely used to increase the bulk of pollen in order to reduce wastage during artificial pollination programmes.

BURSTING OF POLLEN AS A TEST OF VIABILITY

Andronescu (1915) and Kearney and Harrison (1932) describe the bursting of pollen in nutrient solutions as 'pseudo-germination' and 'ejection.' They interpreted this behaviour for determining the viability. Kearney and Harrison (1932) report that the percentage of viability calculated on the basis of 'ejection' was confirmed after artificial pollination and the percentage of seed set. In *Gossypium herbaceum* and *Pennisetum typhoideum* the pollen fails to germinate even after short periods of storage. However, in the latter plant prominent protrusions are formed up to 186 days and the pollen grains burst when placed in the nutrient medium (Vasil, 1958a 1961). In both plants, the stored pollen showed gradual reduction in the percentage of bursting and protrusions. This is comparable to the gradual reduc-

tion in the percentage of germination of stored pollen in other plants.

The evidence presented above leads to the conclusion that the stored pollen which fails to germinate in vitro or only bursts and forms protrusions may not always be non-viable, and if used for pollination, it may give satisfactory fruit or seed set.

The viability of pollen is tested in different ways, but the commonest and most reliable are in the vitro and in vivo germination tests. Occasionally 2,3, 5-tetrazolium chloride (Vieitzev, 1952) is also used but this can be done only in pollen with a thin and colorless exine. Recently King (1960) determined viability on the basis of peroxidase reaction. Acetocarmine is sometimes utilised for this purpose. Bajpai and Lal (1958) considered pollens of several crop plants to be viable as long as they stained with acetocarmine. Unfortunately this criterion is not dependable, since it is well known that fresh as well as preserved pollens, irrespective of whether they are viable or non-viable, often stain alike with acetocarmine (Vasil, 1958c; see also King, 1955). The staining capacity depends not on the viability but on the contents of pollen grains. At times, even herbarium specimens (dead for many years!) have been used to study the nuclei in pollen grains (Leitner, 1938; Khosoo, 1956). Leitner (1938) states that the pollen of *Lolium perenne* taken from herbarium material stained normally, although it is known to lose viability after one day.

ERRATIC GERMINATION OF STORED POLLEN

A major source of difficulty to those engaged in the storage of pollen is the erratic germination of stored samples (see Nebel and Ruttle, 1937). Quite often even if pollen fails to germinate in one or two previous tests, it may give a high percentage of germination in subsequent tests. In some other cases, the percentage of germination after storage for a few days is higher than that of fresh pollen. Such irregularities are "generally found when the pollen is collected from apparently equally mature flowers on different days or in different, though neighbouring, localities, and even when it is taken from flowers at the same age in a given locality and at about the same time" (Holman and Brubaker, 1926). According to Nebel and Ruttle (1937), "these variations may be due to lack of uniformity in the sample, but other unknown factors may possibly be involved."

NUTRITIONAL REQUIREMENTS OF STORED POLLEN AND CAUSES OF LOSS OF VIABILITY

For optimum germination and maximum tube length, the fresh pollen of *Pennisetum typhoideum* vars. T.55, I.C. 1472 and T.25, and of *Arachis hypogaea* var. TMV.2, 115 requires 12.5, 25 and 10 per cent sucrose, respectively; stored pollen of these plants requires 15, 27.5 and 12.5 per cent sucrose (Vasil, 1958a, 1961). Other workers have also observed that, compared to fresh pollen, stored pollen requires higher concentrations of sugar for normal germination.

Kühlwein (1937) made similar observations in some gymnosperms. According to Kühlwein and Anhaeusser (1951), the fresh pollen of *Ceratozamia, Pinus* and *Picea* collected in June gave optimum germination in two per cent sucrose. In the middle of December, stored pollen required 20 per cent sucrose for optimum germination. With the ageing of pollen, from June to August and later to December, higher and higher concentrations of sugar were needed. This behaviour was attributed by the authors to decrease in the permeability of the pollen. Further, there also appears to be a correlation between high sugar concentration and intensity of respiration, which is usually lower in aged pollen. Aged pollen is like a resting cell; to stimulate it to germinate, higher concentrations of sugar are necessary. Visser (1955) has shown that even the boron-sensitivity of pollen increases with age.

As a rule, in most plants pollen loses viability soon after dehiscence of the anthers, especially where temperature and humidity are unusually high. Occasionally viability may be retained for some time even under natural conditions. In *Hordeum* and *Oryza,* fertilization cannot be secured with certainty unless the pollen is transferred directly from the anther to the stigma (Anthony and Harlan, 1920; Nagao and Takano, 1938). There is no seed formation in *Sorghum* when pollinated with five-hours-old pollen (Stephens and Quinby, 1934). Pollen of *Paspalum dilatatum* does not show any germination 30 minutes after dehiscence of the anthers (Bennett, 1959). Similarly the pollen of *Zea mays* stored in pollinating bags in direct sunlight, at 46°C, remained viable for only three hours. On the other hand, pollens of certain fruit trees, e.g., apple, pear and plum (also those of some gymnosperms), retain viability for several weeks or even months.

Naturally the question arises: What are the causes which lead to the loss of viability? It is well known that stored pollen usually becomes

dry and shrivelled, and shows poor germination or none at all. However, it shows better results if exposed to higher humidities for some time before germination and is supplied with higher concentrations of sucrose than those required by fresh pollen. The probable causes of the loss of viability may, therefore, be desiccation, utilization of reserve food and inactivation of enzymes, causing failure of metabolic processes which may be responsible for germination, both in vivo and in vitro (see also Nebel and Ruttle, 1937). So far there is no conclusive proof to support these assumptions, but some indirect evidence has no doubt been put forward from time to time.

Nielsen (1956) reports that the pantothenic acid content of the pollen of *Zea mays, Alnus glutinosa, A. incana* and *Pinus montana* shows a substantial decrease after a year's storage. Pantothenic acid is a vital constituent of coenzyme A which is of paramount importance in respiration and general metabolism of plants. Thus it is likely that its loss during storage, along with the loss or inactivation of certain other vital systems, may be responsible for the failure of germination (Vasil, 1958a, 1961). Further, changes in the color of the oil sticking on the exine of *Lilium* pollen seem to be accompanied by the loss of viability (Pfeiffer, 1955). Knowlton (1922) has studied the correlation between ageing of pollen and loss of moisture, respiration, food depletion, and reduction in the quantity of certain enzymes.

The present state of our knowledge does not justify any definite conclusion, and extensive research work on the chemical composition of fresh and stored pollen grains may be helpful.

CORRELATION BETWEEN VIABILITY OF POLLEN AND FRUIT SET

Quite often stored pollen does not germinate in vitro but produces a satisfactory fruit set in vivo. Occasionally, there may be a poor fruit set or none at all, even if the pollen otherwise gives a moderate germination in vitro (see Sandsten, 1909; Knowlton, 1922; Gollmick, 1942). Failure of germination in vitro may probably be due to the deficiencies caused during storage which are later compensated by the stigmatic and stylar tissues at the time of germination. In support of this view can be cited the work on *Streptocarpus* (Roemer, 1915), date (Albert, 1930), *Pistacia* (Stone et al., 1943), grape (Olmo, 1942), tobacco (Hagiaya, 1949) and tomato (Visser, 1955), where stored pollen fails to germinate or the germination is very poor (sometimes as low as five

per cent) but the fruit set is fairly satisfactory. Pollen of potato stored at –30°F for 7, 12 and 13 months gave no germination or a very poor germination in culture media but when used for artificial pollinations it gave very satisfactory fruit and seed set. Thus, absence of germination in vitro does not necessarily imply that the stored pollen is dead or too weak for field use (King, 1955).

Holman and Brubaker (1926) also believed that pollen which is "incapable of germinating in artificial media may be stimulated by some substances in the stigma to form a tube, and perhaps under favourable conditions, bring about fertilization." Knowlton (1922) had earlier shown that *Antirrhinum* pollen, which remains viable for 670 days, will not germinate in artificial media after 180 days unless a piece of the stigma is placed in the medium. Stored apple pollen which failed to germinate otherwise was induced to give 40 per cent germination in vitro after being kept over 80 per cent R.H. for one week.

Stored pollen is of prime importance to plant breeders, horticulturists and others who use it for large-scale articial pollinations in the field. Whether or not the stored pollen will give a fruit set comparable with that of fresh pollen requires serious consideration. Overley and Bullock (1947) found that apple pollen, which gave 35 per cent germination in vitro produced satisfactory field results. On the basis of pollinations with stored pollen of apple and pear, Visser (1955) suggests the relationships in Table I between percentage of germination in vitro and fruit set:

TABLE I

Germination	Fruit set
Less than 20 per cent	Nil or poor
20 to 40 per cent	Poor to moderate
40 to 60 per cent	Moderate to normal
Over 60 per cent	Normal

However, Visser (1955) states that his "experiments with apple and pear have shown that pollen mixtures containing 98-99% dead pollen and 1-2% living pollen still gave moderate fruit set, provided comparatively large amounts were used for pollination."

In general, pollen grains showing 30–60 per cent germination in culture media produce a normal fruit set. The above investigations are of immense importance, and it would be worthwhile to extend our knowledge, particularly in relation to plants of economic importance.

LONGEVITY OF POLLEN AND TAXONOMIC RELATIONSHIP

By comparing the longevities of different pollens Pfundt (1910) attempted to conclude the taxonomic relationships of the various species studied by him. He pointed out that the more closely related the species, the greater the agreement in the longevity of their pollen. He further stated that "the longevity under the most favourable conditions is ordinarily uniformly long or short in the species of a given circle of relationship, short for example in the Helobiae, Gramineae, Polygonales and Opuntiales, and long in the Pinaceae, Pandanales, Salicaceae, Fagales, Rosales and Primulaceae." Holman and Brubaker (1926), who have dealt with this question in some detail, remarked that "with a few exceptions the conclusions drawn from his (Pfundt, 1910) data are not very convincing." On the basis of the mean longevity of air-dry pollen, they arranged the families as in Table II.

TABLE II

Rank	Family	Longevity in days
1	Amaryllidaceae	38
2	Primulaceae	34
3	Rosaceae	31
4	Leguminosae	28
5	Saxifragaceae	27
6	Ranunculaceae	25
7	Liliaceae	23
8	Salicaceae	21
9	Scrophulariaceae	19
10	Gramineae	1

On the other hand, on the basis of mean longevity of pollen depending on favourable humidity, the ranking was as in Table III.

TABLE III

Rank	Family	Longevity in days
1	Primulaceae	119
2	Leguminosae	115
3	Saxifragaceae	105
4	Rosaceae	101
5	Liliaceae	86
6	Amaryllidaceae	75
7	Salicaceae	71
8	Ranunculaceae	70
9	Scrophulariaceae	69
10	Gramineae	1

While the pollen grains of most of the Gramineae either show very poor germination or fail to germinate in artificial nutrient media, *Pennisetum typhoideum* is an exception (Vasil, 1958a, 1960b, 1961). In this plant (vars. T. 55,I.C.1472 and T.25) the pollen remained viable for 186 days over concentrated sulphuric acid (0 per cent R.H.) at 16°–35°C. Over other humidities, too, they were viable for similar periods, but at higher humidities (60 per cent and above) viability was limited to only five days. The percentage of in vitro germination (78 per cent) and tube lengths (4,320 microns) were also excellent.

From the foregoing account it is obvious that under low temperature and humidity, pollen usually retains viability for longer periods, although requirements vary from plant to plant. Generally, the near-freezing temperatures and 25–50 per cent R.H. are suitable for prolonging longevity. Temperatures as low as –180° or –190°C, obtained by using liquid air or liquid oxygen, respectively, appear to be ideal but are far too expensive and require a costly and elaborate set-up.

What factors are responsible for the loss of viability during storage are not fully understood at present.

CULTURE

INTRODUCTION

Giovanni Batista Amici (1824, 1830), an Italian astronomer and mathematician, while examining the papillate stigma of *Portulaca olera-*

cea, noticed that one of the 'hairs' terminated in a pollen grain. This was the discovery of the pollen tube. He also saw a pollen grain giving out a tube which entered the stigmatic tissues and gradually disappeared in the style. Ultimately, in 1830, he concluded that the pollen tubes elongate, bit by bit, and finally come in contact with the ovules (see also Maheshwari, 1950).

Horticulturists and plant breeders often fail to get fertile seeds in spite of all the care taken during artificial pollination. Unless sterility is the main cause, failure of seed setting may be due to slow growth of the pollen tube or its early degeneration in the style. By removing the stigma and part of the style and placing a drop of a sugar-agar-gelatin medium (suitable for the germination of the pollen of various species of *Solanum*) on the cut end of the style, Swaminathan (1955) obtained viable seeds of the cross *Solanum pinnatisectum* with *S. lanciforme* as well as with *S. bulbocastanum* which do not cross otherwise.

The earliest observations on pollen tube growth appear to be those of Von Mohl (1834) who noticed that in a saturated humid atmosphere pollen grains of some plants readily produced tubes. Later, Schleiden (1849), Van Tieghem (1869) and others studied the in vitro germination of pollen grains of several plants and concluded that the germination varied from species to species or even from variety to variety.

Van Tieghem (1869), Lidforss (1896, 1909) and Jost (1905, 1907) reported that the pollen grains of *Dactylis* and *Hippeastrum* germinated readily in water. Knight (1917), Martin and Yocum (1918), Schoch-Bodmer (1936) and Righter (1939) were able to germinate the pollen of *Corylus, Pinus* and *Pyrus* even in distilled water. Daniel (1952), Savelli and Caruso (1940) and Paton (1921) claim to have obtained good pollen tube growth in water alone (in *Impatiens, Nicotiana* and *Plantago*). On the other hand, Cooper (1939), Schwarzenbach (1953), Vasil (1958a, 1960a) and most other workers state that merely in water, germination is usually poor and the tubes are short.

Normally pollen grains do not germinate satisfactorily in water, but aqueous solutions of sucrose (occasionally other sugars also), with or without addition of accessory substances, produce good results (see Vasil, 1958a, 1960a). Pollens of some plants may germinate easily under a wide range of conditions, while in others the requirements may be very exacting, failing which there may be no germination. As a rule,

the length of the pollen tube obtained in vitro is significantly shorter than that in vivo. Consequently, one of the main problems is to obtain in vitro germination and tube length comparable to that in vivo. In a few cases the length of the tubes in cultures does equal that in nature, e.g., *Pyrus* (Knight, 1917), *Rumex* (Schoch-Bodmer, 1921), *Chionodoxa, Hippeastrum, Muscari, Puschkinia* and *Scilla* (Brink, 1924c), *Vitis* (Branscheidt, 1929, 1930), *Convallaria, Echeveria, Gagea, Genista, Impatiens, Pachyphyllum, Ribes, Scilla, Sedum, Tradescantia, Vicia, Vinca* and *Xanthosoma* (Ehlers, 1951) and *Pennisetum typhoideum* (Vasil, 1960b).

During recent years pollen grains have been increasingly used for physiological studies because they have a simple structure and lend themselves easily to metabolic investigations. As they are highly sensitive to external factors, particularly temperature and humidity, the effects of these factors on pollen germination and tube growth can be readily ascertained. However, the pollen grains show great variability when they are collected from different anthers of a flower or even from the same anther (see Brink, 1942a; Smith, 1942; Daniel, 1952; Kubo, 1954; King, 1955; Vasil, 1958a, 1960a; Kurtz and Liverman, 1958). It is not surprising that the diploid and tetraploid pollen grains (of *Cucumis melo*) also require different media for their optimal germination (Tanaka and Mukai, 1955). Therefore, apart from the other usual precautions, one requires uniform samples, collected under normal environmental conditions. Since the quantity and density also affect germination and tube length, approximately an equal number of pollen grains have to be spread evenly on the medium for obtaining comparable results (see Brink, 1924d; Visser, 1955).

It has been the experience of many workers that in spite of these and other precautions, there is always some variation. Even under apparently similar conditons, pollen grains germinated in the same nutrient medium on two successive days may fail to give identical results. These variations can, however, be largely attributed to the inherent variability of the pollen grains and the climatic conditions prevailing during the growth and flowering of the plant. With the change of season also, the germinability of pollen grains varies and they require higher concentrations of sucrose and boric acid. Sometimes marked differences are noticed if the pollen of the same species is cultured at two places, especially when one of them happens to be in a tropical country and the other in a temperate one.

ROLE OF SUGARS

Most pollen grains germinate successfully in sugar solutions. Sucrose has largely been used, but lactose (Bishop, 1949), dextrose (Faull, 1955) and several other sugars and sugar derivatives (O'Kelley, 1955; Raghavan and Baruah, 1956a; Vasil, 1958a, 1960a) have also been tried. According to Vasil (1958a) sucrose, dextrose, rhamnose, raffinose, lactose and galactose generally give good results, while fructose, mannose and mannitol are unsatisfactory. Sometimes galactose, dextrose and lactose serve as well as sucrose. The utilisation of lactose by germinating pollen grains is of considerable interest, since its presence in plants has not yet been conclusively demonstrated (Pigmann and Goepp, 1948). Kuhn and Löw (1949a, b) have recorded the presence of lactose in the pollen of *Forsythia;* this is perhaps the only record of its kind. In contrast, fructose—which is so common in plants—usually fails to produce satisfactory germination or tube length. It appears that the suitability of a particular sugar need not necessarily depend on its natural occurrence in plants.

It is well known that autotrophic plants or plant tissues which contain chlorophyll are able to manufacture carbohydrates on which their growth and development depend. In the absence of chlorophyll they become parasitic or saprophytic and derive their nutrition from other sources. As a rule, pollen grains do not contain chlorophyll (except in a few cases, e.g., in the Malvaceae; see Maheshwari, 1950) and are, therefore, dependent on external sources for the supply of essential nutrients. At the time of shedding, they do contain some reserve food materials which are utilised during the initial stages of germination but are not adequate for the enormous growth of the tubes which occurs in vivo or in vitro. As stated by Brink (1924a), the reserve food may be sufficient to support some amount of growth which could proceed until it is consumed. However, since the nutrients in the culture medium stimulate and improve the germination of pollen and elongation of the tubes, it indicates that this effect, partly if not completely, is due to the externally supplied nutrition.

In a recent personal communication to one of us (I.K.V.), Professor R. A. Brink (University of Wisconsin, Madison, Wis., U.S.A.) stated that "It is difficult to avoid the conclusion, it seems to me, that sugars serve as nutrients during pollen tube growth. The pollen tube formed in many species is a massive structure relative to the reserve

materials stored in the pollen grain, and the reserves often are quickly consumed. Where then are the materials for the pollen tube wall and for energy purposes to come if not from sugars of outside origin?"

Since the beginning of this century, the question whether the pollen tubes growing in vivo and in vitro utilize externally supplied nutrients has attracted much attention. The two main views on this question concern the endogenous or the exogenous utilisation of nutrients. The former school believes that the externally supplied sugars have only an osmotic role and are not utilized by the tube for any nutritional purposes (Jost, 1905, 1907; Martin, 1913; Anthony and Harlan, 1920; Visser, 1955). Adherents of exogenous nutrition point out that, apart from having an osmotic role, the externally supplied sugars in the medium (or in the style) definitely serve as a nutrient material for the growing tubes (Brink, 1924a; O'Kelley, 1955, 1957; Hellmers and Machlis, 1956; Vasil, 1958a, 1960a).

According to Visser (1955), the "presence of sugar is only essential for creating favourable osmotic conditions for germination" and "many pollen may germinate readily and produce tubes of considerable length in pure water or on substrata which do not contain sugar." In support, Visser (1955) quotes the work of Ehlers (1951) who cultivated pollen grains of different genera without any external source of nutrition in the medium and obtained tube lengths which were sufficient to effect fertilization. Visser concludes: "none of the observations with regard to exogenous nutrition of pollen tube can be regarded as valid proof" and believes that "the pollen tube is exclusively built up from the reserves of the pollen grain" and the "effect of sugar on germination is in all probability exclusively due to its osmotic properties in aqueous solutions."

Visser (1955) further mentions that "pollen germination depends among others on the rate at which water is released from the medium and taken up by the pollen." He calls this phenomenon the "diffusion rate of water" and points out that for "controlling the diffusion rate of water" the presence of sugars is essential in order "to create a certain osmotic value." He continues: "the growth of the tube of many different pollens, whether cultivated in vivo or in vitro, is independent of the presence of nutrients in the medium in the form of sugars." The supporters of endogenous nutrition of pollen tubes believe that, even in vivo, the tubes do not get any nourishment from the style (see Visser, 1955). However, Dr. Ir. T. Visser (Tea Research Institute,

Talawakelle, Ceylon) recently wrote (to I.K.V.), after the publication of O'Kelley's work, that largely the externally supplied sugars do serve as a source of nutrition for the germination of pollen, though in some cases it may be a 'luxury' consumption, if taken up from external sources.

In *Hippeastrum aulicum,* Jost (1907) obtained 17,000 to 22,000-microns-long tubes in one per cent sucrose, but when the concentration was reduced to 0.25 or 0.5 per cent the length of the tubes was only 7,000 to 8,000 microns. In three per cent sucrose, Brink (1924a) obtained pollen tubes growing beyond 10,000 microns. In one to ten per cent sucrose the pollen grains of *Vinca minor* produced tubes 10,000 microns long (Bobilioff-Preisser, 1917; Brink, 1924a). Equally long tubes have been obtained with the pollen of *Nicotiana alata* and *Scilla* (Brink, 1924a), and with *Crotalaria juncea* and *Dolichos lablab* (Vasil, 1958a). In *Pennisetum typhoideum* the tube length (4320 microns) in vitro is sufficient to effect fertilization in vivo (Vasil, 1960b).

Further evidence concerning exogenous utilization of sugar, although indirect, comes from the experiments of several workers, e.g., Dodel-Port and Dodel-Port (1880), Mangin (1886), Green (1894a, b), Tanaka (1955, 1956) and Konar (1958), who observed that the pollen grains of different species (specially gymnosperms) show abundant starch when placed in sugar solutions. In *Typha latifolia,* pollen tubes grown on sucrose solutions gradually accumulate oil globules (Vasil, 1958a).

Brink (1924a) and Schoch-Bodmer and Huber (1947) noticed that when pollen was cultivated in sugar or sugar-agar media, the tubes were as long as, or even longer than, those formed in nature. In many plants germination is no doubt high but the tubes are considerably shorter than those formed in vivo, even when the medium is supplemented with different growth-promoting substances. However, this could be due to the shortage of certain essential growth-promoting substances in vitro. On the other hand, when the medium lacks sugar, there is profuse bursting of pollen grains and often no germination at all.

Although no attempts have been made to determine the dry matter of pollen before and after germination, it is obvious that it increases considerably during germination. This can be visualised from the massiveness of the tubes and the large number of callose plugs formed in them. Brink (1924a) states: "Tubes are formed with cellulose or

callose walls reaching a length in some cases equal to several hundred diameters of the pollen grain and containing in their frequently bulbous tips a considerably enhanced mass of protoplasm. These tubes, moreover, commonly form numerous callose plugs the total volume of which may alone exceed that of the grain. The inference is clear that the sugars in the medium have been drawn up in the development of these structures."

The studies of O'Kelley (1955) on the pollen tubes of *Tecoma radicans* gave the final blow to the theory of endogenous nutrition. He used C^{14}-labelled sugars and conclusively proved, for the first time, that pollen tubes respire during their growth and absorb externally supplied sucrose, fructose or glucose. To quote his own words: "The specific activity of the CO_2 produced showed that during pollen tube respiration of individual sugar 36 per cent of the CO_2 came from the glucose, or 66 per cent came from the fructose, or 72 per cent came from sucrose." Experimenting with the pollen grains of *Pinus ponderosa,* Hellmers and Machlis (1956) noticed that the pollen grains "absorb and metabolize a variety of mono-, di- and tri-saccharide sugars. These are synthesized into polysaccharides and are respired. Further, pollen germinated and supplied with sugar continues to be active and healthy when pollen dependent on endogenous reserves has obviously begun to die." In a more recent paper, O'Kelley (1957) has provided further evidence to show that the externally supplied sugars are undoubtedly utilised.

Tupý (1960) has also demonstrated the utilization of exogenous sucrose by the growing pollen tubes of apple and *Nicotiana alata* and the enzymatic inversion of sucrose into glucose and fructose (see also Iwanami, 1959). The fact that inversion of sucrose stops when the pollen tubes are removed from the culture medium indicates that invertase is released by the tubes into the medium. The breakdown of sucrose takes place either within the tube or on its surface as in yeasts and somatic cells of higher plants. When boron is added to the medium, the inversion of sucrose is quicker. Another point which Tupý (1960) has emphasized is that growth of pollen tubes is not very satisfactory in the inversion products of sucrose, viz., glucose and fructose (this is in conformity with the findings of some earlier workers; see Vasil, 1960a). It may be assumed that pollen grains absorb sugars mainly in the form of sucrose.

According to Iwanami (1959), at the time of shedding, pollen

grains usually contain sucrose, glucose or fructose as food reserves. If the pollen contains mainly glucose (e.g., *Impatiens*), it germinates earlier than those containing sucrose (e.g., *Lilium*). In the latter case sucrose is converted into glucose just before germination. Iwanami points out that at the time of germination in most pollen grains starch is converted into sugars, or vice versa. This regulates the osmotic pressure of the pollen grain in relation to the osmotic pressure of the medium or the tissues of the stigma and style. Whether it is a general phenomenon in pollen grains requires further investigation.

As early as 1889, Correns computed that the reserve food present in the pollen was not adequate to produce a pollen tube long enough to reach the ovules. It is well known that the conducting tissue of the style contains considerable quantities of starch, sugar and other carbohydrates (Brink, 1924a; Gessner, 1948). Straub (1947) concluded that in *Petunia* the pollen tubes are nourished by the conducting tissue during their growth through the style. Schoch-Bodmer (1945) and Schoch-Bodmer and Huber (1947) have also made similar observations. Linskens (1954; see also Bhattacharjya and Linskens, 1955) has recently demonstrated that during their growth down the style, pollen tubes not only absorb water but also take up quite an appreciable amount of sugar. After heavily pollinating the mature stigmas of *Petunia*, Linskens noticed that after the pollen tubes had reached the embryo sac, the sugar content of the styles was reduced to about half the amount that was present before pollination. During their growth towards the ovary pollen tubes of *Lilium* and *Petunia* also utilise exogenous substrates from the style (Linskens and Esser, 1959).

Concerning the role of sugar as an osmotic agent, Brink (1924a) writes: "A source of no little annoyance and perplexity to those who have attempted to cultivate pollen artificially has been the bursting of pollen and pollen tubes." There is profuse bursting in lower concentrations, and it would be interesting to know whether it is dependent on osmotic concentrations. According to Van Tieghem (1869), Molisch (1893) and Lidforss (1896), bursting can not be an osmotic phenomenon, since no distinct relationship is known between the number of pollen grains bursting and the concentration of sugar in the medium. Lloyd (1918) believed that bursting is entirely due to imbibition by the protoplast beyond the strength of the wall. Waddington (1929) holds that bursting of *Matthiola* pollen is in no way influenced by sugar concentration. However, his data clearly show that

the percentage of bursting was much lower in higher concentrations of sucrose than in lower. Bursting is inversely related to the osmotic concentration of the medium and sometimes it can be reduced by adding agar or gelatin to the sucrose medium (Anthony and Harlan, 1920; Walderdorf, 1924; Schoch-Bodmer, 1936; Kuhn, 1938; Bair and Loomis, 1941; Schwanitz, 1942). Vasil (1958a, b, 1960a) altered the osmotic concentration of the medium by adding different molar concentrations of mannitol (an inert substance which does not have any promoting effect on the germination of pollen) and showed that bursting of the pollen of *Cucumis melo* var. *utilissimus* and *Momordica charantia* invariably decreased with an increase in the osmotic concentration of the medium, and vice versa.

There is general unanimity of opinion on the role of sugars in controlling osmotic concentration during germination of pollen. That it does affect germination itself is also obvious, and probably an approximate similarity in the osmotic concentration of the medium with that of the pollen is a prerequisite for good germination. According to Schoch-Bodmer (1936), turgor pressure is very largely responsible for the germination of pollen and may also affect the length of pollen tubes. However, germination is not an osmotic or a turgor phenomenon alone, as shown conclusively by the work of O'Kelley (1955) and Hellmers and Machlis (1956), nor does sugar serve merely as a source of nutrition.

ROLE OF BORON

Since the very beginning most investigators have attempted to improve the germination of pollen grains and the length of tubes in vitro. During the years 1932, 1933 and 1935, Schmucker made an important discovery and published a series of papers from Göttingen on the stimulating effect of boric acid. He observed that pollen of the tropical species of *Nymphaea* hardly germinated in one per cent glucose, but that satisfactory germination resulted if stigmatic extract was added to it. After analyzing the stigmatic extract, he noticed that it contained appreciable quantities of boric acid. This led him successfully to germinate the pollen of *Nymphaea* in one per cent glucose supplemented with boric acid. On quantitative analysis it was found that the pollen grains required almost the same concentration of boric acid as was present in the stigmatic secretion. Therefore, he concluded that boron plays a strategic role in the germination of pollen. He fur-

ther observed that 0.001 or 0.01 per cent boric acid promoted growth of the tubes not only of *Nymphaea* but also of a large number of other plants. Among the 40 species tested, ten showed marked sensitivity, and boric acid not only raised the percentage of germination but also accelerated the rate of growth of the tube. Schmucker was so much impressed by the stimulating action of boric acid that he designated it as an "anorganischer Wuchsstoff" (inorganic growth substance). He thought that boric acid was probably bound with hydroxyl-rich organic substances, like sugars, and formed boron-hydroxyl complexes which were related to the formation of cell wall material.

The stigmatic secretion of *Vitis* also contains a boric acid concentration equivalent to that required for optimal germination of pollen in vitro (Gärtel, 1952). Thomas (1952) also identified boron in the styles of *Lilium* and demonstrated its stimulating action on *Lilium* pollen in vitro.

Several workers have pointed out that ten ppm (0.001 per cent) or higher concentrations of boric acid are toxic to plant growth (see Schmucker, 1935; Visser, 1955). However, pollen grains can tolerate concentrations up to 1,200 ppm (0.12 per cent), although optimum results are obtained between ten and 150 ppm (0.001 to 0.015 per cent). Vasil (1958a, b, 1960a), who investigated the effect of various concentrations of boric acid (also borax) on the pollen of a number of crop plants, reports that 100 to 150 ppm (0.01 to 0.015 per cent) of boric acid is favourable for pollen germination, while higher concentrations are definitely inhibitory. The effect on elongation of the tube is much more marked than on the percentage of germination. In certain plants, e.g., *Capsicum annuum, Corchorus capsularis* and *Crotalaria juncea,* optimum germination and tube length were obtained in sucrose solutions alone, and addition of boric acid either had no effect or proved inhibitory. In these plants the natural level of borin appeared to be adequate and, therefore, any additional supply proved ineffective.

Pollen is said to be naturally deficient in boron (see O'Kelley, 1957) but the pollen grains and styles of at least some plants have a high boron content (Bertrand and Silberstein, 1938; Bobko and Zerling, 1938; Gärtel, 1952; Thomas, 1952). Even in boron-deficient pollen, the deficiency is likely to be made up by the boron from the style.

It may be argued that boric acid prevents the growth of microorganisms and also affects the pH of the medium. Vasil's (1958a) data do not confirm this view. Boric acid, in the concentrations generally

used for pollen culture, neither changes the pH of the medium nor is effective in checking the growth of the microorganisms which appear in the medium after six to eight hours of culture.

Another role of boron may be an interaction with certain substances which affect germination. Two views have been expressed, one by Gauch and Duggar in 1953 (see also Gauch and Duggar, 1954; Linskens, 1955; Sisler et al., 1956; Baker et al., 1956; O'Kelley, 1957) and the other by Visser in 1955.

While conducting experiments on the respiration of root tips of lima bean and pea, Gauch and Duggar (1953) made some interesting observations and proposed that "boron combines with sugar to form a sugar-borate complex (ionizable) which is translocated with greater facility than are non-borated, non-ionized sugar molecules." Linskens (1955) has now demonstrated experimentally that, in *Petunia,* sugar-borate complexes are formed during pollen germination. Gauch and Duggar (1953) pointed out two possibilities how boron affects the translocation of sucrose or its hydrolytic products. Firstly, "the borate ion could react with sucrose (or glucose or fructose), the sugar then passing through the cellular membranes as the ionized sugar-borate complex until such time as a cell utilizes this complex and liberates the boron ion." Secondly, it is also "possible that the borate ion is associated with the cellular membranes, that it there reacts chemically with the sugar molecule facilitating its passage through the membrane, and that the sugar is freed on the inside of the cell by a second reaction." They further state that the plants "absorb boron throughout their life cycle, indicating that, as new cell membranes are formed, more boron is required." Boron has also been shown to be relatively immobile in plants (Eaton, 1940; see also Kuhn, 1943). Thus the evidence is in favour of the second possibility, that the borate ion is associated with the cellular membranes.

In addition to boron, under certain conditions, some other factors may also limit the absorption and translocation of sucrose. According to Gauch and Duggar (1953) boron appears to be "the dominant factor in the movement of sucrose (or its hydrolytic products) from cell to cell in the plant"; and what holds good for plants in general, seems to be a pertinent explanation of the role of boron in pollen germination. However, as observed by Visser (1955) and Vasil (1958a, b, 1960a), boron shows some stimulating effect on the germination of pollen and elongation of tubes even when the pollen grains are ger-

minated in water without any supply of sugars. So far, there does not seem to be any satisfactory explanation for this behaviour.

Schmucker's (1935) suggestion that the beneficial effect of boron is due to the fact that it regulates the hydration of colloids, is associated with polyhydroxyl compounds of the membrane, and is concerned in the formation of pectic substances, has been further substantiated by the experiments of Dennis (1937), Minarik and Shive (1939), Kuhn (1943), Baker et al. (1956), Münzer (1960) and others. Spurr (1957) has recently shown that in boron-deficient plants the cell walls are markedly thinner than in those where boron supply is adequate. His experiments indicate that boron is a "morphogenetic agent affecting the development of specific form of the cell wall of the plant."

The other view was put forward by Visser (1955), who made detailed investigations on the effect of boric acid on the germination of pear and apple pollen, and who suggested that boron plays an important role in its carbohydrate metabolism. He observed that the in vitro germination of pollen improved when boron (as aqueous solution of boric acid) was fed to the tree branches. Germination could also be stimulated by feeding sugar to the branches. However, optimum germination occurred in pollen collected from branches fed with sugar as well as boric acid. Therefore, Visser concluded that boron increases the vitality of pollen. It was also seen that the germinability of pollen is the same, "whether the 'extra' boron is taken up from the tree beforehand or taken up by the pollen from its germination medium afterwards" and "the degree of boron sensitivity of the pollen is inversely related to the boron level of the plant."

Bobko and Zerling (1938) observed higher viability in the pollen of *Trifolium pratense* when the plants were given boron-rich fertilizers. Antles (1951) reports that the pollen of pear which failed to germinate in sugar solutions without boric acid, gave 100 per cent germination in sugar alone when the plants were given boron-rich fertilizers for a period of four years. Batjer and Thompson (1949) obtained a higher fruit set by spraying boric acid on pear blossoms.

According to Visser (1955), the length of the tube is directly related to the concentration of boric acid, while the percentage of bursting is inversely related. The formation of protrusions through the germ pores is independent of boric acid supply, but their subsequent growth depends on its presence. In Visser's own words "boric acid is specifically required for the growth of the pollen tube." This is contra-

dicted by Vasil's (1958a, 1960) experiments where he has shown a greater increase in the percentage of germination in media containing boric acid than in those where boron had been omitted.

Visser (1955) also pointed out that the "necessity of a continuous and ample supply of boric acid implied that it is inactivated in the course of the elongation process." He emphasized that "On account of the favourable effect of boric acid on tube growth it can be assumed that a complex of boric acid and an unknown substance acts as a 'growth promoter' in tube elongation. . . . It is suggested, therefore, that the substance in question is partly present in a free form, termed X, and in a complex form with boric acid, termed XB; X has no germination properties, while XB has. The proportion of X and XB is determined by the boron level of the plant. On account of the above observations the following view can be held with respect to the increase of germination and tube growth upon addition of boric acid: Whenever the growth rate of the tube (as determined by sugar concentration and temperature) exceeds the supply of the complex compound from endogenous sources, this supply can be supplemented by addition of boric acid, thus leading to additional complex formation with the available free compound . . . Finally, it can be remarked that the substance X is possibly a glucoside." The higher percentage of germination seen when large groups of pollen grains are cultured in one drop, or by the addition of pollen extracts, or mutual stimulation of pollen, is also due to the presence of the substances X and XB. Loss of viability during storage of pollen and an increased boron-sensitivity of the stored pollen are due to the immobility of the substance XB.

Tupý (1960) has shown that "Boron encourages sucrose absorption proportionately to the stimulation of pollen tube growth. It is, therefore, possible that the stimulatory effect of boron on growth is connected with carbohydrate metabolism. In view of the fact that pollen tube growth is accelerated by boron at the time when the tubes are still drawing to a great extent or exclusively on the reserve substances in the pollen grain — and in view of the well known stimulatory effect of boron in distilled water — it follows that the main factor is not necessarily the translocation of carbohydrates into the pollen tubes, but that it is rather a question of the rate of their metabolism."

In a recent paper on the germination of pollen of *Tecoma radicans*, O'Kelley (1957) reports that addition of boron brought about a 24

per cent increase in sugar absorption over the controls grown without boron. According to him the effect of boron on pollen tube growth is "not very closely related" to borate effects on either respiration or sugar absorption, but "It appears that boron has a specific role in pollen tube growth, in addition to a stimulatory effect on oxygen uptake and on sugar absorption." The stimulation of tube growth may involve the synthesis of pectic materials required for wall formation of the elongating tube.

The effect of boron far surpasses the effect produced by any known hormone, vitamin or chemical. This is perhaps due to the fact that pollen grains initially contain adequate quantities of growth-promoting substances and that, therefore, any additional supply does not improve germination. On the other hand, pollen seems to be usually deficient in boron, and its addition to the nutrient medium improves not only the germination but also the elongation of the tubes.

On the basis of present evidence, the role of boron in pollen germination and pollen tube growth may be three-fold (Vasil, 1960a): (*a*) it promotes absorption and metabolism of sugars by forming sugar-borate complexes, (*b*) it increases oxygen uptake, and (c) it is involved in the synthesis of pectic materials for the wall of the actively elongating pollen tube.

Daniel and Váróczy (1957) believe that "boron is promoting the movement and availability of the reserve materials and stimulating the activity of the adenosine-triphosphate; however, the possibility of some pure physico-chemical effect of boron might also be taken in consideration."

Moewus (1950) claims that in *Forsythia intermedia* self-sterility could be overcome by the application of boric acid. Kuhn (1943) had pointed out earlier that boric acid plays the role of an inactivator for germination-inhibiting substances in tomato pollen. However, Moewus' (1950) work on the breaking of self-sterility in *Forsythia* has been shown to be incorrect by Esser and Straub (1954), Visser (1956) and Reznik (1957). It may be added that Moewus and Visser both worked on the same bushes of *Forsythia* but obtained entirely different results.

EFFECTS OF CHEMICALS, GROWTH SUBSTANCES, VITAMINS, ANTIBIOTICS AND CAROTENOIDS

Besides sugars and boric acid, several other substances are also known to have a stimulatory effect on the germination of pollen and

the growth of tubes. Lidforss (1896) observed that, even in small amounts, sodium chloride, potassium nitrate and calcium nitrate proved toxic to pollen grains, and Brink (1924b) has confirmed these results. However, according to several recent reports, it appears that calcium nitrate and manganese sulphate, as well as several other chemicals, improve the germination and elongation of pollen tubes (Loo and Hwang, 1944; Sen and Varma, 1955b; Raghavan and Baruah, 1956b, 1959; Takami, 1956; Vasil, 1958a, b, 1960a). Branscheidt (1930) and Gotoh (1931) traced the effect of these ions to the changes induced in the pH of the medium.

Hormones and vitamins have often been used to increase the percentage of germination and to accelerate the rate of growth of tubes (Dandliker et al., 1938; Smith, 1939, 1942; Addicott, 1943; Loo and Hwang, 1944; Antles, 1949; Anhaeusser, 1953; Dikshit, 1956; Sen and Varma, 1956b, 1958; Vasil, 1958a, b, 1960a). Contrary to the general belief, in many cases these substances do not produce a marked effect on the germination of pollen grains (see Vasil, 1958a; Rietsema, 1961). However, Brink (1924b) obtained a substantial increase in *Cucumis sativus, Lythrum salicaria* and *Primula obconica* by addition of 'sterile' yeast (which is a source of vitamins of the B group) to the nutrient medium. In this connection Cooper's (1939) observations are of interest. He states that the influence of several vitamins and amino acids on the pollen of *Carica papaya* appears to be due to their regulating effect on the pH.

Addition of aspartic acid, glutamic acid, histidine or cysteine to the sugar-agar medium causes germination of *Paris hexaphylla* pollen which otherwise fails to germinate (Sawada, 1958). Similarly, arginine, valine and alanine promote germination of *Oryza sativa* pollen (Sawada, 1958). The above amino acids also occur in the pollen grains and pistils of these plants.

Schwarzenbach (1953) investigated the effect of 25 carotenoids on the germination of *Cyclamen persicum* pollen and noticed that, while a few stimulated germination, three carotenoids (also present in the anther and pollen of *C. persicum*) inhibited germination. Therefore, he suggested that carotenoids of the anthers prevent a premature germination of pollen. When the pollen lands on the stigma, the inhibiting effect "is removed and transformed to one of stimulation by an unknown substance" from the unfertilized ovules. This unknown substance can be replaced in experiments by indoleacetic acid and vitamin K_1.

Curtis and Duncan (1947) point out that orchid pollen contains enough auxin for normal germination, while in *Antirrhinum* and *Bryophyllum* the auxin content is said to be a limiting factor for germination (Smith, 1942).

Occasionally addition of traces of penicillin and streptomycin (the latter is frequently contaminated with phenylacetic acid) to the culture medium improves the germination and growth of pollen tubes (Pulvertaft, 1946; Vasil, 1958a, b, 1960a; Sen, 1960). In some plants antibiotics are known to cause inhibition of growth (Rosen, 1957).

During the last decade, two new chemicals — kinetin and gibberellic acid or gibberellins—have been reported to show a marked effect on the growth of plants and plant tissues (Miller et al., 1955a, b, 1956; Brian 1957; Skoog and Miller, 1957; Stowe and Yamaki, 1957; Vasil, 1957; Stodola, 1958). In *Lilium,* gibberellic acid greatly accelerates the rate of elongation of the pollen tubes (Kato, 1955; see also Chandler, 1957; Stodola, 1958). Chandler (1957) observed that pollen, which did not germinate on a control medium and appeared non-viable, germinated satisfactorily with the addition of gibberellic acid. She presumed that, due to the stimulating effect of gibberellic acid on the pollen of *Lilium* and *Petunia,* it may be possible to "overcome some of the physiological incompatabilities characteristic of certain clones of these species." Whereas gibberellic acid promotes the germination of *Tulipa* pollen, IAA has no effect. However, in conjunction with gibberellic acid IAA has some beneficial effect (Laboureur, 1960; see also Bose, 1959, Ching and Ching, 1959).

So far, the effect of kinetin has not been tried on the pollen of angiosperms, but it is likely that its incorporation in nutrient media along with auxins may hasten the division of the generative cell. Konar (1958) reports that in *Pinus roxburghii,* kinetin alone (i.e., without any added auxin) promotes germination and increases the tube length, but nuclear divisions are not induced.

EFFECT OF pH

Although the influence of pH on the growth and metabolism of plants in nature as well as in cultures has been proved beyond doubt, there is not much work on the effect of pH on the germination of pollen. Opinions are so conflicting that it is difficult to decide whether pH at all influences the germination of pollen (Vasil and Bose, 1959).

As early as 1924, Gotoh pointed out that the dissolved alkali of the cover glass greatly affected the germination of pollen. A little later,

Brink (1925) observed that in *Lathyrus odoratus* optimum germination occurs at a pH of 7.0 and that the zone of hydrogen-ion concentration favourable for germination is rather narrow. He assumes that the pH may modify the growth of pollen tubes through a direct effect on the chemical reactions attending the digestion of reserve food materials. In *Petunia,* optimum germination occurred at a pH of 5.5 to 5.8. In several plants, Berg (1930) and Sisa (1930) obtained double-optima curves (two-peaked) expressing the percentage of germination at different pH (see also Branscheidt, 1930). Vasil and Bose (1959) observed that the pH of the medium remains almost unchanged, even after the pollen tubes have grown in it for one to two hours.

Satisfactory germination of pollen has been reported at pH 4–9 (Berg, 1930, Branscheidt, 1930; King and Johnston, 1958). In certain crop plants the percentage of germination and the length of the pollen tubes remain fairly constant at pH 3.5 to 9.0 (Vasil and Bose, 1959). The optimum pH range suitable for the germination of *Cosmos bipinnatus* pollen changes about every 20 days during the flowering season (Kubo, 1954). Pollen grains of several species of *Rhododendron* germinate only at pH 3 during the beginning and at the end of the flowering season, but during the middle of the season they germinate in a wide range (pH 1.8–7.8). Throughout the flowering season the optimum pH value gradually extended from the acidic to the alkaline range (Kubo, 1955a, b). The pH may not directly affect germination but whatever effect is produced may be entirely due to the cations and anions present in the medium in the form of buffer salts. Therefore, it has been suggested that instead of using different buffer mixtures, N/10 HCl or N/10 NaOH may be used for adjusting the pH of the medium from 3.5 to 9.0. Addition of buffer mixtures makes it almost impossible to avoid and estimate the effect of different salts present therein.

EFFECT OF TEMPERATURE

The correlation between temperature and pollen germination is usually represented by an optimum curve (Roberts and Struckmeyer, 1948; Visser, 1955), and maximum germination and tube length are obtained between 20° and 30°C (Winkler, 1926; Berg, 1930; King and Johnston, 1958). In tobasco variety of red peppers, Hirose (1957) reported optimum germination of pollen at 35°–40°C which is rather unusual since in other plants germination is inhibited at these tem-

peratures. At low temperatures the percentage of germination and tube length are considerably reduced, and under such conditions in vivo the tubes may never reach the ovules (Smith and Cochran, 1935). At sub-optimal temperatures, satisfactory tube lengths can be obtained only if the period of growth is not a limiting factor. Adams (1916) reported 90–100 per cent germination in apple at 14°C after 24 hours. However, at 2°C, Östlind (1945) observed only 10–36 per cent germination after 69 hours, but it shot up to 90–100 per cent after 119 hours.

The diameter of the pollen tubes enhances with increase in the environmental temperature (Smith, 1942). For example, the pollen of *Antirrhinum* showed negligible growth at 15°C, optimum germination and tube length at 25°C, pronounced broadening and 'bloating' (swelling) of the distal portion of the tube at 30°C, and extensive bursting of the bloated tubes at 35°C. On the other hand, in *Bryophyllum* appreciable germination occurred at 15°C and optimum germination and tube length at 25°C. Smith mentions that "At 35°C a great diversity of tube lengths resulted with many short, broad tubes and some exremely long ones with broadened proximal portions." In *Medicago sativa* (Sexsmith and Fryer, 1943), for a half-hour growth period, a linear relationship was observed between the length of the pollen tube and rise in temperature. Thus the length increased as the temperature was raised from 70° to 100°F. There was no germination at 50°F but at 100°F the pollen tubes appeared to be normal. The Q_{10} is approximately two (Visser, 1955; Vasil, 1958a).

Sen and Varma (1956a) report that in *Pisum sativum*, "as compared to room temperature (10°–12°C) increased germination and elongation of pollen tubes was obtained at 15° to 21°C. At temperatures below 12°C the germination was comparatively poor, while at 24° the pollen tubes burst profusely." In *Areca catechu* optimum germination was noticed at 28°C, whereas at 15°, 20°, 30° and 35°C germination was usually poor (Raghavan and Baruah, 1956a). In *Dolichos lablab, Pisum sativum* and other crop plants, Vasil and Bose (1959) observed optimum germination and elongation of pollen tubes at 25°–30°C. At 5°C and below there was practically no germination, and it was inhibited above 35°C. At higher temperatures marked swelling or bursting of the tips was quite common. Irrespective of the temperature, the growth curve of the tube remains typically sigmoid (Vasil, 1958a; Vasil and Bose, 1959).

CHEMOTROPISM AND GROUPING OF POLLEN

The chemotropic reaction in vitro of pollen tubes to pistil has been widely described. Van Tieghem (1869) regarded that pollen tubes are attracted towards the ovary by hydrotropism, while Strasburger (1878) found no evidence of chemotropism towards ovules. Molisch (1893) noticed that the pollen tubes of *Narcissus tazetta* are negatively aerotropic; e.g., only the pollen grains near the periphery of the culture medium germinated and sent their tubes toward the centre. Miyoshi (1894) claims to have seen drops of fluid at the micropyle and believes that these secretions are responsible for attracting the pollen tubes in a number of plants. Chemotropism towards the stigma has since long been considered a common phenomenon (Miyoshi, 1894; Lidforss, 1896).

Recent studies indicate that, of a large number of species examined, only a few showed positive chemotropism towards their own pistils or other floral parts (Brink, 1924d; Gotoh, 1931; Savelli and Caruso, 1940; Tsao, 1949; Ustinova, 1954; Visser, 1955; Schneider, 1956; Iwanami, 1959). According to Miki (1955; see also Miki, 1954), "In *Camellia sinensis,* pollen tubes show positive tropism to the fresh style slices while they show negative tropism to slices of the styles which are steamed for 10 minutes at 60°C, 80°C or 99°C." and "both substances, which are responsible for the positive and the negative tropism, diffuse from styles to agar media." Slices of fresh, mature stigma and of the subjacent tissue stimulate pollen germination in *Lilium longiflorum* (Rosen, 1959). They exhibit chemotropism which is lost on heating, washing and exposure to air.

The aggregation of pollen grains in the culture medium (as well as on the stigma) has a marked stimulatory effect on the germination and the length of tubes. This can be compared with the 'bios effect' of yeasts and other microorganisms where the growth of the organism is stimulated when the inoculum is heavy. The stimulating[1] effect on pollen is presumably due to certain growth-stimulating substances which diffuse out of the pollen grains (Brink, 1924d; Kuhn, 1938;

[1] In a recent conversation, Dr. W. R. Tulecke drew my (BMJ) attention to the forthcoming article by B. H. Kwack and J. L. Brewbaker (Amer. Jour. Bot.—In Press), pointing out that the stimulation, at least in the 15 species of plants tested by them, is due to calcium ion. Although calcium is not replaceable by ions such as strontium, other cations (Mg^{++}, K^{+} or Na^{+}) are required to permit the calcium activity.

Savelli, 1940; Savelli and Caruso, 1940; Beck and Joly, 1941; Holubinsky, 1945; Rémy, 1953; Visser, 1955; Ariyasu, 1959; Iwanami, 1959). However, the effective concentration of these substances occurs only when the pollen grains are aggregated. Visser (1955) states that "The experiments with an increasing number of grains per drop and those with pollen extracts imply that a substance (or substances) with 'germination promoting' properties diffuses from the grains" and that this substance is neither boric acid nor an enzyme but perhaps a glucoside.

Our understanding of the stimulatory effects due to the grouping of pollen and chemotropism of pollen tubes must await further researches.

EFFECT OF PLANT TISSUE EXTRACTS

The earlier reports on the chemotropic influence of pistil on pollen tubes initiated studies on the effect of extracts of various other floral parts. East and Park (1917, 1918) showed that the in vitro growth of pollen tubes of *Nicotiana* is promoted by stigmatic extracts. According to Knowlton (1922), even stored pollen, which otherwise failed to germinate in vitro, was stimulated to germinate with addition of small crushed pieces of the stigma to the culture medium. Tokugawa (1914) and Brink (1924d) obtained similar results in several other plants, e.g., *Narcissus*. In some cases even the crushed ovarian tissue and extracts of ovules and pollen proved stimulatory (see Brink, 1924d; Visser, 1955). Kuhn (1938) pointed out that anther extracts considerably raised the germination percentage and the length of tubes.

As early as 1926 Katz stated that the stigmatic secretion is indispensable for pollination but maintained that it merely protects the pollen from drying and probably does not act as a chemical stimulant. Kühlwein (1948) observed that the extracts of anther, pollen or stigma generally increased the percentage of germination and the length of the pollen tube. Addition of pollen extracts of *Tulipa,* and anther and stigmatic extracts of *Lilium,* greatly increased the percentage of germination as well as tube length in *L. candidum*. A few reports also indicate that the extracts of stigma, style or pollen either have no appreciable effect on germination or are inhibitory (Sasaki, 1919; Kaienberg, 1950; Sen and Varma, 1955a).

Takashima (1954) noticed that in the Cucurbitaceae "On the flowering day, a certain hormone generates in the stigma and that hormone promotes the growth of the pollen tube."

Kuhn (1938) mentions that in *Matthiola* a considerable increase in the percentage of germination and the length of tubes occurs by the addition of anther extract to the medium, and he assumes that the effective substances of the extract belong to the pollen. The promoting effect could not be replaced by yeast extract, small amount of sugar or diastase. High concentrations of the extract inhibited germination.

Visser (1955) also records some stimulation on the percentage of germination as well as on tube length, but generally it "is in no case as high as that in the sugar solution to which boron had been added."

GROWTH PHENOMENA

It has already been said that the growth of pollen tubes in vitro is characterised by a typical sigmoid curve. Even when pollen grains are germinated under different conditions, the pattern of growth remains unaltered. The maximum rate of elongation occurs some time before the middle of the growth period. Active elongation of the tube takes place only in the region of its tip (Schoch-Bodmer, 1945; Haeckel, 1951; O'Kelley and Carr, 1954; Mühlethaler and Linskens, 1956; Iwanami, 1959).

The recent work of Tulecke (1953, 1957, 1959) on the in vitro production of tissue masses from the pollen grains of *Ginkgo biloba* opens up a new field of study in the physiology of pollen. So far this is the only instance of such a behaviour but it is likely that angiosperm pollen would also respond similarly.

PROTOPLASMIC STREAMING. During the course of their growth, the pollen tubes of almost all angiosperms show rapid protoplasmic streaming. It begins within a few minutes after the pollen is sown in the culture medium or lands on a stigma and continues until the pollen tube has ceased to elongate. The rate of protoplasmic streaming varies from plant to plant and is directly proportional to the rate of elongation of the tube (see Vasil, 1958a, b, 1960a). The rate of movement (in vitro) is slow in the beginning, gradually increases and comes to a peak slightly before the middle of the growth period, and then gradually declines until the pollen tube stops elongating. Occasionally, however, the streaming movement may continue for some time even after the tube has ceased to elongate. In an actively growing pollen tube the rate of streaming is rather slow near the pollen-grain end, is fairly rapid in the rest of the tube, and there is practically no stream-

ing in the extreme tip which has been termed as the 'cap block' by Iwanami (1956).

In gymnosperms, where growth of the pollen tube is extremely slow, the streaming of the cytoplasm has never been demonstrated (see Takeuchi, 1953). Even in some angiosperms, where the rate of elongation of the tubes is poor, the cytoplasmic streaming is either very slow or not at all visible, e.g., in *Citrus* and some species of *Solanum* (Vasil, 1958a).

Iwanami (1956, 1959) has described several types (e.g., rotation, circulation, fountain, contra-fountain etc.) of protoplasmic streaming in pollen tubes. In fact, such variations may occur at different places in the same tube or in different tubes in the same culture.

Patterns of Pollen Tube. During in vitro growth under abnormal conditions, the tubes often exhibit characteristic patterns in shape which may be due to the pollen being out-of-season or old, or to high concentrations of the nutrient medium. The tube may be bloated—enormous swelling of the tip; blunt — abrupt and flattened tip; or spirally coiled like a cork-screw (see Hartmann-Dick and Müller-Stoll, 1955). Pollen collected during high atmospheric humidity and temperature shows similar behaviour.

Occasionally another interesting behaviour known as 'vesicle formation' has been described. Actively growing pollen tubes abstrict small vesicles at their tip. These vesicles have an independent existence for some time, show cytoplasmic streaming and finally degenerate (Bobilioff-Priesser, 1917; Vasil, 1958a). Sometimes the vesicle may contain one or both the sperm cells (Bobilioff-Preisser, 1917). Similar observations have recently been made by Iwanami (1959) in *Lilium longiflorum*. In *Brassica nigra* the formation of vesicles could be stimulated if the pollen was sown in a high concentration (35 per cent) of sucrose (Vasil, 1958a).

Callose Plugs. As the pollen tube elongates (in vitro), certain plug-like structures are formed at regular intervals all along its length and divide it into small segments. These plugs are made up of callose, a substance very similar to cellulose (Müller-Stoll and Lerch, 1957; Currier, 1957). Callose is a polyglucoside derived from glucose but its exact chemical nature is still uncertain (see Currier, 1957). Its formation depends on the limited capacity of pollen tubes to utilize the glucopyranose component of the metabolised sucrose during respiration (Tupý, 1960). This explains the increased accumulation of cal-

lose in incompatible and slow-growing tubes (Tupý, 1959; Vasil, 1960a).

Callose plugs also appear in tubes growing in vivo except in those cases where the tubes remain rather short. The pollen tubes of some plants may develop callose plugs in vivo but not in vitro, e.g., in cotton (Iyengar, 1938; Vasil, 1958a).

With the formation of successive callose plugs, the cytoplasm and nuclei move towards the tip of the tube. The latter is capable of independent existence, and it has been experimentally demonstrated in *Vinca minor* that the excised tip continues its growth normally (Brink, 1924d; see also Iwanami, 1959). The portion of the tube next to the pollen grain becomes isolated from the actively growing tip. It does not contain any nuclei, and the cytoplasm is also scanty. Gradually, as the tube elongates, the older portions continue to collapse. The formation of callose plugs not only gives mechanical strength to the tube but also restricts the protoplasmic streaming to the actively growing region. The cytoplasm between the two callose plugs may also show streaming movement for some time but finally it stops.

After the pollen tube has attained its maximum length, its wall becomes thickened and may sometimes appear like a fibre (Vasil, 1958a, b, 1960a).

Experiments with self-sterile *Nicotiana alata* and Lord Lambourne variety of apple showed that if pollen tubes failed to grow due to incompatibility, there was at least twice as much callose per unit length as in normally growing compatible tubes (Tupý, 1959). In apple this was primarily due to the greater length of the plugs, but in tobacco this was also due to their greater density. In incompatible tubes callose formation is not a primary result of incompatibility but is directly related to the inhibition of their growth. Vasil (1958a, 1960a) has demonstrated that in certain members of the Cucurbitaceae, after the pollen tubes cease to elongate, there is a heavy deposition of callose in the tubes both in vitro and in vivo.

CHEMISTRY

INTRODUCTION

As compared to our knowledge of the storage and culture of pollen, very little work has been done on the chemistry of pollen. The major drawback in this field is the difficulty in securing sufficient quantities

of uniform samples, and consequently studies have been restricted to plants producing relatively large amounts of pollen e.g., *Pinus* and *Zea mays*. Nevertheless, the chemistry of pollen is engaging attention partly because of its importance for physiological studies and partly due to its nutritional and medicinal value.

Pollen grains are of particular interest to the entomologist because they are the chief source of all nutrition, except water and perhaps carbohydrates, required by bees. The nutritional value depends on their chemical contents—inorganic elements, essential amino acids, vitamins, hormones and enzymes. A Russian botanist (Tsitsin; The Sunday Express, London, April 15, 1945) claimed that the longevity of most of the centenarians in Russia depended on their diet of honey or honey scrap (a thick layer of almost pure pollen at the base of the beehive).

Usually the pollen samples are obtained from pellets deposited by bees while passing through pollen traps (see Vivino and Palmer, 1944; Auclair and Jamieson, 1948). The pollen collected by bees is largely from a particular plant species, and there is very little mixture with foreign pollen. However, bee-collected pollen does show some difference as compared to hand-collected pollen from the same plant. This is partly due to certain changes occurring in the bee-pollen and also due to contamination with nectar and honey. Pollen stored in the beehive undergoes lactic acid fermentation and gives rise to the so-called bee-bread.

In 1954 Lundén published "A Short Introduction to the Literature on Pollen Chemistry" which summarises most of the important contributions on the subject. We have, therefore included only selected titles for review on this subject.

PROTEINS AND AMINO ACIDS

Medical men are interested in isolating pollen proteins which are probably the cause of pollen-allergy. Bee-keepers are also interested in this aspect with a view to develop a suitable food for rearing bees.

The technique of chromatography has proved very helpful in the qualitative and quantitative analysis of the amino acids of pollen proteins. Their amount varies from species to species and may be as high as 35 per cent of the total content (Todd and Bretherick, 1942). Almost all the common amino acids have been obtained from pollen grains, and they may occur either in a free state or bound to proteins (Auclair

and Jamieson, 1948; Sarkar et al., 1949; Nielsen et al., 1955; Takashima, 1955; Virtanen and Kari, 1955; Varma and Varma, 1956; Iwanami, 1959). However, the presence of phenylalanine, tryptophane, hydroxyproline, tyrosine and aminobutyric acid is not very common. The amino acid composition of pollen proteins and that of soybean flour, casein and whole egg powder does not show any marked differences (Weaver and Kuiken, 1951).

Considerable work has been done on the protein fractions isolated from pollen, and proteins of comparatively low molecular weight (about 5,000) containing carbohydrates have been obtained (see Lundén, 1954). Free proteins and those conjugated to carbohydrates or pigments, e.g., artefolin, trifidin and pratensin, have been isolated and purified. Nucleoproteins have also been reported in birch pollen (see Lundén, 1954).

CARBOHYDRATES

At various stages of development, pollen grains normally show considerable quantities of starch but invariably it disappears during cell divisions or at the time of shedding. In the first case it is due to the utilization of starch in the laying down of the new cell wall while its disappearance just before the dehiscence of anthers is said to be due to its conversion into sugars (Iwanami, 1959).

Carbohydrates have been frequently detected in protein fractions of pollen extracts, and the presence of starch and fat has been reported in innumerable embryological publications (see Tischler, 1917; Luxemburgowa, 1928; Maheshwari, 1950). Ribose and desoxyribose are invariably associated with nucleoproteins. Kuhn and Löw (1949a, b) reported lactose in the pollen of *Forsythia,* which happens to be the only authentic record of its presence in the plant kingdom. Some pollen also contains cellulose, pentosan and reducing sugars. The protein, carbohydrate and reducing sugar content of the pollen of *Pinus montana, Alnus glutinosa, A. incana* and *Zea mays* has been recently determined by Nielsen et al. (1955). In such studies the mode of collection of pollen is an important factor. For example, bee-collected pollen usually contains large quantities of reducing sugars partly because of the presence of honey or nectar in the cementing fluid, and partly because of the high humidity at which the pollen pellets are stored, which causes enzymatic breakdown resulting in a decreased content of non-reducing sugars. On the other hand mechanically collected and

air-dried pollen is rich in non-reducing and poor in reducing sugars (Todd and Bretherick, 1942).

Quadrio (1928) and Mameli (1952) report that generally the anemophilous plants (wind-pollinated) have starchy pollen, while pollen of entomophilous plants (insect-pollinated) is rich in fat and sugar which serve as nutrients for the insects. According to Mameli (1952), there is an association between fatty pollen and porogamy (pollen tube entering through the micropyle), and between starchy pollen and aporogamy (pollen tube following any other path than entering through the micropyle). Such an association does not necessarily hold good since in some plants having starchy pollen the tube does enter through the micropyle (see Maheshwari, 1950).

VITAMINS AND GROWTH-PROMOTING AND GROWTH-INHIBITING SUBSTANCES

The pollen grains of gymnosperms usually have low vitamin content, whereas those of angiosperms are rich in vitamins of the B group but poor in fat-soluble vitamins (Lundén, 1954). Biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, thiamin, ascorbic acid and vitamins A, D, E and K have been reported in the pollen of maize, date, plum and many other plants (see also Ridi and Aboul Wafa, 1950; Weygand and Hofmann, 1950; Nielsen et al., 1955). Pollen grains appear to be highly nutritive materials as far as water-soluble vitamins are concerned. The curative effect of corn pollen on avian polyneuritis was proved by Dutcher as far back as 1918.

Folic acid (also folic acid conjugates and folic acid conjugases) has been reported in the pollen of *Pinus montana, Phleum pratense, Secale cereale* and *Zea mays* (Nielsen and Holmström, 1957). The occurrence of folic acid is of considerable interest, since it "is believed to be an important component of the so-called vitamin T which is reported to be an important growth factor for insects" (Nielsen and Holmström, 1957).

Kakhidze and Medvedyeva (1956) have recently pointed out that the pollen of tobacco excretes considerable quantities of vitamins into the artificial germinating medium. Probably these are liberated even in vivo and are absorbed by the stylar tissue.

In 1956 Nielsen showed that while all other vitamins from the pollen of *Pinus montana, Alnus glutinosa, A. incana* and *Zea mays* did not

show any appreciable change after a year's storage (in a cool, dry place), the pantothenic acid content had substantially decreased in all the samples.

Oestrone and an oestrogenic substance have been detected in the pollen of *Salix* and date palm (see Lundén, 1954; Hassan and Aboul Wafa, 1947). Growth-promoting substances giving positive responses to *Avena*-test are known to occur in the pollen of a large number of plants: maize (Mitchell and Whitehead, 1941; Wittwer, 1943; Redemann, 1951), orchids (Müller, 1953), apple (Larsen and Tung, 1950) and *Pinus densiflora* (Tanaka, 1958). In the acid fraction of corn pollen, Fukui et al. (1958) have reported 3-indoleacetic acid and two other unidentified growth-promoting substances.

Besides the growth-promoting substances, growth inhibitors have also been occasionally reported (Larsen and Tung, 1950; Tanaka, 1958). Tanaka (1958) believes that the slow growth of the pollen tube of *Pinus densiflora* may be due to the presence of inhibitors in its own pollen.

ENZYMES

It is now generally accepted, especially after the publication of O'Kelley's (1955) work, that pollen tubes metabolise externally supplied sugars both in vitro and in vivo. It is also known that the pollen tubes digest the food reserve while passing through the tissues of the style, and this can be possible only if they secrete enzymes. As expected, several enzymes and coenzymes have been reported by different authors in the pollen of many plants, e.g., *Pinus, Digitalis, Fritillaria, Lilium, Nicotiana, Portulaca,* maize, rice, rye and apple (see Paton, 1921; Branscheidt, 1930; Kühlwein, 1937; Haeckel, 1951; Venkatasubramanian, 1953; Lundén, 1954).

Okunuki (1939, 1942, 1943) studied the repiration and coenzyme content of nine pollens, and concluded that the alcoholic fermentation brought about by pollen was identical with that caused by yeast. Haeckel (1951) made extensive studies on the phosphatase, amylase and saccharase activities of the pollen of *Digitalis, Fritillaria, Lilium,* etc. and found that the phosphatase activity was generally as high as in seeds. Palumbo (1953) recently investigated the content of succinic acid dehydrogenase, acid and alkali phosphatase and adenosinetriphosphate in the developing pollen of *Lilium longiflorum* and *Tradescantia paludosa.*

The more important conclusions of Haeckel (1951) are:

(*a*) There is a correlation between the stylar structure and the enzymatic activity. If a stylar canal is present, e.g., in many monocotyledons, the pollen tube does not come in intimate contact with the stylar tissue; therefore, pollen enzymes (amylase or invertase) act only extra-cellularly. When the styles are solid and possess a conducting tissue, e.g., in many dicotyledons, large quantities of phosphatase are found in the pollen. However, there is no correlation between the length of the style and the degree of enzymatic action.

(*b*) The enzymatic activity of the pollen diminishes with ageing and is sometimes as low as two-thirds to three-fourths of the original level. Enzymatic activity also drops from the influence of ultra-violet light, humidity and temperature. Under extremely low temperatures (−190°C) the enzymatic activity can be preserved without any loss for an "indefinite" period; therefore the pollen retains its viability for an equally long period (Bredemann et al., 1947).

(*c*) The amylase present in pollen not only hydrolyses starch but also synthesizes starch from the sugar present in the nutrient medium (Green, 1894a; Branscheidt, 1930; Kühlwein, 1937). According to Branscheidt (1930), it also promotes growth of the pollen tube.

(*d*) Invertase hydrolyses sucrose into monosaccharides which during further enzymatic breakdown liberate energy necessary for the growth of the pollen tube. It also helps in the osmo-regulation necessary for the elongation of the tube.

(*e*) Phosphatase probably plays a role in nuclear division. As a rule, the extreme tip of the pollen tube does not show any phosphatase activity. Although phosphatase is no doubt necessary for elongation of the pollen tube, it bears no relation to the rate of growth.

(*f*) During pollen germination, phosphatase, amylase and invertase activity rise considerably, sometimes as much as 600 per cent.

MISCELLANEOUS NOTES

Several ether-extractable materials, like saturated hydrocarbons, higher alcohols, sterols and fatty acids, also occur in pollen grains (see Lundén, 1954). A number of pigments, mainly flavonols and carotenoids, are responsible for their coloring. Insect-carried pollen is generally rich in carotenes but these are absent in wind-carried pollen.

Pollen grains invariably contain large quantities of pollenin, the

substance forming the thickened exine which makes the pollen 'resistant' to environmental and other factors.

The ash content of pollen usually varies from one to seven per cent and may reveal potassium, magnesium, calcium, copper, iron, silicon, phosphorus, sulphur, manganese and titanium.

Schwarzenbach (1955, 1956, 1957) has recently shown that the germination of pollen grains is often inhibited if the medium includes serum of human patients who have once suffered from jaundice, cancer, carcinoma and sarcoma. Sera of normal human beings or those suffering from other diseases (so far tested) does not affect the germination. In this field our knowledge is very much limited at present, but in due course it may be possible to utilize the inhibitory action of the sera of diseased persons for the detection of various diseases.

Further studies on the chemistry of pollen will undoubtedly prove useful in understanding the causes of loss of viability during storage, for controlling diseases due to pollen-allergy, for detecting some serious diseases, and above all for developing a highly concentrated and nutritious food for human beings.

SUMMARY AND CONCLUSIONS

The present review largely deals with the storage and culture of pollen. A few pages have also been devoted to the chemistry of pollen. Further, attention has been drawn to the utilization of such investigations in plant breeding and horticulture.

In order to retain viability for prolonged periods, a near-freezing temperature and a relative humidity of 25–35 per cent are ideal. The longest period of viability (3287 days at −17° to −37°C) is reported for the pollen of *Pyrus communis* and *P. malus* (Ushirozawa and Shibukawa, 1951). Loss of viability during storage may be due to utilization of stored food materials, desiccation and inactivation of certain vital systems like the enzymes. Stored pollen requires higher concentrations of sugar for proper germination, and if germination is about 35 per cent in vitro, it may result in normal fruit set in the field. In some cases, stored pollen, which may apparently be dead, revives if kept for a few days over comparatively high humidities.

In the U.S.A. and some countries in Europe it is now a standard practice to use stored pollen for artificial pollinations. It would be worthwhile to establish 'pollen banks' in every country so that the

pollen of economically important plants could be available throughout the year.

Sugars act as nutritive materials for the germination of pollen as well as for the growth of the tube. However, for proper germination a near-similarity in the osmotic concentration of the nutrient medium and that of the pollen is a prerequisite. The percentage of germination and the length of tubes are directly proportional to osmotic concentration, while bursting is inversely proportional.

The effect of boron on pollen germination and on elongation of tubes is much more marked than the effect of any known hormone, vitamin or chemical. It promotes absorption of sugars and their metabolism by forming sugar-borate complexes, increases oxygen uptake and is involved in the synthesis of pectic materials required for the wall of the actively elongating pollen tube. Pollen of most species seems to be naturally deficient in boron.

Hormones, vitamins, carotenoids, antibiotics and several other inorganic salts help to improve germination in many cases. Recently, a new chemical, gibberellic acid, has also been shown to have a marked effect on the elongation of pollen tube. It is likely that pollen contains adequate quantities of vitamins and hormones; therefore, their addition to the nutrient medium generally does not markedly improve germination and tube length.

Pollen grains germinate in a wide range of pH, but best results are obtained in pH 5.5 to 6.5, and the pH of the medium remains almost unchanged even after the tubes have grown in it for one to two hours. Under normal conditions the pollen of most plants shows optimum germination between 20°–30°C, and the Q_{10} is approximately 2. Higher temperatures cause bursting of pollen and the pollen tubes show abnormal forms.

Pollen tubes of several plants exhibit positive chemotropism towards their own floral parts and sometimes to floral organs of other plants, too. Grouping of pollen grains causes higher percentage of germination and better elongation of the tubes. This seems to be due to the excretion of certain 'growth-promoting' substances from the pollen grains themselves. Extracts of pollen, ovules, ovaries, styles and stigma also stimulate germination.

The growth curves of pollen tubes in vitro are typically sigmoid and do not undergo any alteration, even if the temperature or the nutrients are changed. The pollen tubes of angiosperms usually show rapid pro-

toplasmic streaming which is directly proportional to the rate of elongation of the tube. Callose plugs are commonly formed in the tubes. Under abnormal conditions pollen tubes exhibit various types of deformities.

Pollen grains contain several inorganic substances, carbohydrates, proteins, vitamins, growth-promoting substances and enzymes. Studies on the chemistry of pollen are of vital importance for bee-keeping, treatment of allergies and the development of a nutritious and concentrated food.

In conclusion, it may be pointed out that studies on the physiology of pollen offer great possibilities for overcoming barriers to crossability; understanding the effect of pollination on the growth of fruit; inducing parthenogenesis; obtaining parthenocarpic fruits; increasing fruit set; treatment of pollen allergies and in the detection of some serious human diseases.

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SEEDLING MORPHOLOGY IN APPLIED GENETICS AND PLANT BREEDING

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SEEDLING TECHNIQUES

A seedling is "a plant produced from seed in distinction to a plant propagated artificially," according to Jackson (1900). This definition is not altogether satisfactory, as it does not take age into account. Hence the definition by Kerner and Oliver (1894) of a seedling as "a young plant raised from seed" is preferable and will be used here.

John Beale wrote in 1657 that: "I find the Truth, and that much more might be added to Lawson's Rules of distinguishing the Hopefulness of the Fruit by the first leaves of the yearling plant: For a short and dark green leaf prognosticateth a crabbed Fruit: With a larger leaf, and thick, but also dark green, I have found a good Winter Apple, but the Stock hardy, to endure a stiff Clay-ground: A fady willowish broad leaf noteth a flat insipid apple, as the *Kentish Codling* which holds out well against Blasts. A Paler Green, as the *Popinjay,* or *Barbary Leaf,* especially if the leaf be limber also, noteth a delicate fruit; and the broader the Leaf the fairer that Fruit; a wrinkled leaf, neither very dark, nor very light, proves red-streak'd, Crab stocks have reddish Tops. More particulars will occur by ordinary observations".

Later Poiteau (1827) wrote that: "In sowing the seeds of kernel fruits for new varieties, after the plants have come up and grown a year or two, it is common to select for preservation, with a view to fruiting, such as have few or no spines, large leaves, and thick shoots;

such plants, especially among pear seedlings, for the most part produce summer fruits of a small size and little flavour". M. Van Mons, on the contrary, "chooses thorny plants, in which the spines are long, and furnished with buds to their summit, and of which the general aspect of the plant recalls to mind some good known variety. When these plants bear fruit, he sows their seeds, and again the seeds of the fruit so produced to the 4th, 5th, and 6th generation. The peach and apricot sown in this manner have not produced excellent fruit till the third generation, the apple till the fourth generation, and the pear till the fifth or sixth generation. It is to be observed, that out of each generation a choice of plants is made on the same principles as out of the first".

Much later Luther Burbank (1849-1926) in the United States was able to find at least a few characters in seedlings that proved to be correlated with adult characteristics (Howard, 1945). However, his extravagant claims were reduced by Shull (loc. cit.) who, although recognizing that there were some correlations, realised that these were very general and not specific. The basic correlation was that "if the seedling shows lush growth, thick stems and fat smooth buds, the subsequent growth of such plant can be expected to give large and better fruit than if the seedlings show a tendency to have thin, spindly stems, and narrow buds".

De Vries (1907), who was acquainted with Burbank's work, recognized that there could be correlations between seeds, seedlings and adult plants. Thus a dark coloured seed indicates a brightly coloured flower, whereas a pale seed is usually associated with white or pale corollas. Stocks, for instance, have greyish, brown or bluish seeds, according to the colour of their petals, and red and blue lupins may often have dark seeds, but the white variety *(Lupinus angustifolius)* has white seeds. White varieties generally have yellow or greenish seeds, in contrast to dark seeds of the common species. There are other examples with a relationship between flower colour and seed colour.

Seedling techniques involving these kinds of correlations have not yet been widely used in applied genetics, such as modern plant breeding. For instance, in a survey of plant breeding at the John Innes Horticultural Institution, the only seedling technique mentioned by Haskell (1950) is in connection with his selecting cold hardy lines of sweet corn *(Zea mays)*. Similarly, the History of the Plant Breeding Station at Svalof mentions only freezing experiments (–15°C to –20°C

for 4 days) with winter wheat seedlings still in seedboxes for improving hardiness (Akerman, 1948), and the use of vegetative reproduction at the seedling stage. This method can be a useful technique for building up stocks of clones of the most promising seedlings, and is especially valuable after colchicine treatment of cereals. In the production of wheat-rye hybrids, for instance, the F_1 seeds are germinated, and coleoptiles of seedlings are submerged in 0.25% colchicine solution for about 35 minutes. Repeated divisions are taken from surviving seedlings, and these clones later are transplanted to the field (Muntzing, 1948).

Plant breeders have already used seedling techniques for breeding improved disease-resistant strains of cultivated plants, especially cereals (cf. Table I). Such tests are usually performed on the F_2 generation from the original cross. Generally there is good agreement between seedling and mature plant reactions, as in stem rust and crown rust reaction of oats. Complications may arise, however, as in the use of seedling inoculations for sorting out strains of wheat resistant to yellow rust. If the seedlings are resistant, the mature plants are resistant, but if they are susceptible, then the mature plants may or may not be susceptible (Batts, 1953). Hence it may be advisable later to check seedling behaviour when the plants are adult; even so, there is the possibility of considerably reducing the number of plants for field testing.

Comparative seedling morphology has, however, been of very little interest to botanists since Lubbock's (1896) classic work on the subject. (In some branches of the science, such as fossil botany, it has been ignored, possibly from failure of delicate seedlings to be preserved. Even evolutionists have failed to study this vital stage in the life cycle of a plant.) Modern field botanists are, however, once again reviving interest in seedlings, especially since the introduction of growth-promoting substances used as weed killers. Early seedling stages are often very similar within genera, as in the four British wild species of *Juncus* and *Saxifraga*. Yet sometimes in nature it is possible to recognize seedlings of closely related species, although after studying the seedlings of 233 American plant species, Kummer (1941) concluded that no one seedling character distinguishes an entire family. There is often lack of constancy within a species. Table II shows some genera with species separable at the seedling stage. Seedling taxonomy, so far relatively important, may therefore offer some possibilities for separat-

TABLE I

SOME CULTIVATED CROPS WHOSE SEEDLINGS HAVE BEEN USED FOR DISCRIMINATION IN PLANT IMPROVEMENT FOR DISEASE RESISTANCE

Selection for	Crop	Test Method	Author
Crown rust and stem rust	Oats	Seedling inoculation and isolating rust-free lines.	Kehr et al, 1952.
Cold tolerance	Maize	Seedlings germinated at 50°F.	Haskell, 1948.
Stem rust	Barley	——	Reid, 1938. Brookins, 1940.
Mildew	Grapes	——	Bauer, 1933.
Downy mildew	Soybeans	Seedlings inoculated with spore suspension.	Geeseman, 1952.
Blight resistance	Spinach	Cotyledonary inoculation.	Webb, 1955.

TABLE II

SEEDLING MORPHOLOGY IN SOME SPECIES OF BRITISH GENERA, DISTINGUISHABLE AT THE SEEDLING STAGE

Genus	Species	Distinguishing Characters of Seedlings	Author in "Biological Flora"
Urtica	*dioica* *urens*	Seedlings similar, but *urens* cotyledons ovate instead of oblong.	Greig-Smith, 1948.
Sonchus	*oleraceus* *asper*	Distinguishable by form of first few seedling leaves and laminas.	Lewin, 1948.
Rhamnus	*cathartica* *frangula* (= *Frangula alnus*)	Germination is epigeal v. hypogeal.	Godwin, 1943.
Acer	*pseudo-platanus* *campestre*	Seedlings similar, but those of *campestre* smaller, with thinner instead of more ovate cotyledons.	Jones, 1945.
Nasturtium	*officinale* *microphyllum* (Boenningh ex Rchb.)	Seedlings very similar, but those of *microphyllum* have somewhat more slender hypocotyl and grow more slowly. In later stages seedlings of *microphyllum* have a more prostrate habit.	Howard and Lyon, 1952.
Polygonum	*persicaria* *lapathifolium* *petecticale*	*persicaria* seedlings distinguished by lack of glands and tomentum. The other two species inseparable.	Simmonds, 1945.

ing species. Distinguishing characters can include the shapes of cotyledons or first few leaves, and mode of germinating.

There are many economic examples of varietal identification from seedling characters. These become important to the seed analyst when seeds of closely related varieties are similar, and help to avoid having experimental trial plots to prove correct labelling. One particular method is the use of fluorescent light for separating perennial ryegrass *(Lolium perenne)* from Italian ryegrass *(L. multiflorum),* which has fluorescent properties under ultra-violet light. This method may have wider application than is realised at present. There are other examples of identification based on seedling characters. Van der Vaart (1952) and Morris (1953) have been found that leaf scales from the first and second nodes of pea seedlings at the third or fourth leaf stages are valuable for identifying commercial varieties of peas *(Pisum sativum)* and field peas *(P. arvense).* This method may also be helpful in checking synonyms, and for detecting seed mixtures or mistaken nomenclature. Differences already shown in early development between *P. arvense* and *sativum,* and between varieties in the types, may possibly apply to other crops. There is also the hydrocyanic acid test, used on seedlings of various clovers shortly after their cotyledons have become green to determine whether they are cyanogenetic (see Brett, 1944); in this respect dutch clover *(Trifolium repens)* is acyanogenetic, and wild white is invariably cyanogenetic. Similarly, white clover strains show a good correlation between glucoside content, measured by the picric acid test, and agronomic type determined by plot trials. Indeed, the New Zealand Department of Agriculture uses the picric acid test for clover strain identification in its seed certification scheme (Corkill, 1952).

Identification is often important in species of *Brassica,* as mistakes in stocks of seeds readily occur owing to their similarity. Musil (1950) has provided a key for separating botanical varieties (not horticultural strains) of *Brassica* that uses cotyledon and first internode characteristics of fortnight-old seedlings. Here the main value of the cotyledon is to divide the cultivated forms into two groups of those with large and with small cotyledons. This classification is highly artificial and bears no relationship to the chromosome numbers of varieties within the groups.

It is clear, then, that seedling techniques not only have applications in horticulture and agriculture, but offer the plant breeder a novel

means of selecting from large populations which he could not attempt if they were adults. This article discusses one of these techniques, seedling morphology. It is not intended to be exhaustive, but hopes to draw attention to a neglected field in applied genetics by providing examples chosen to illustrate where it has already proved of help in research. Efficacy of seedling selection depends, of course, on various factors; an important one is whether the material is heterozygous or homozygous, i.e., from more or less outcrossed or from inbred parentage. It is generally necessary to hybridise by outcrossing as widely as possible before using seedling selection, especially for inbreeding species.

SEEDLINGS IN GENETICAL EXPERIMENTS

Among the earliest repetitions of Mendel's investigations of inheritance, Gregory (1911) noted that one of the first natural mutations, called "ivy-leaf" and found in Chinese primrose *(Primula sinensis)*, was distinguishable in the seedling stage, and later he was able to study inheritance of leaf shapes from classification made on segregating seedlings. Yet he overlooked the value of seedlings in classifying for stem and root colour. Altenburg (1916) studied linkage in Chinese primrose, using the characters style length, stigma colour and flower colour, so that all plants had to be grown on to flowering. The value of seedling morphology was mostly overlooked in these typical early genetic experiments.

Classification of adult plants from morphology of seedlings of different ages is not always applicable, as seen in the dwarf mutant of *Portulaca grandiflora*. Blakeslee (1920) noted that normal plants have red stems correlated with red and other colours of flowers, but typical adult dwarf plants always have green stems. Both normal and dwarf young seedlings have red primary stems if destined to produce coloured flowers, but dwarfs have no red in their stems beyond the seedling stage. Dwarfs are, therefore, peculiar in having an inhibitor of colour in the stem, though not inhibiting colours of the flowers. Hence classification may be incorrect at some stages of growth but correct at others.

There are various practical advantages of using a plant like maize for genetical experiments. One is that characters of endosperm and embryo permit examination of segregating progenies in the kernel ('seed'); the characters of the endosperm act as an index to the heredity of an embryo of the same kernel (cf. Eyster, 1934). The use of seed

and fruit characters, as in the study of xenia effects on 'seeds' from irradiation of maize pollen, will not, however, be discussed here. Furthermore, many characteristics of maize are expressed at the seedling stage; hence, for genetic tests large numbers of individuals can be grown in little space, and results arrived at early. Eyster (1934) has discussed the use of experimental cultures of maize seedlings; particular plant characters sometimes can be recognized easily at this stage. Lethals, like white and yellow seedlings, soon die and disappear, but in contrast some characters, such as virescent seedlings and yellow flecked leaves, can be identified only while seedlings, as they show only during the first few weeks of growth.

Seedlings with chlorophyll variations often occur after self-pollination in the progeny of outbreeding crops like maize. One group is the so-called "virescent" type of seedlings of maize. These are yellowish-white, and Demerec (1924) has shown that five genes control five genetically different virescent types. These have proved useful in studying effects of environment on chlorophyll development; high temperature was found to have a stimulating effect on chlorophyll production. Virescents in the early seedling stage are usually without chlorophyll but eventually become green, depending on environmental conditions; if there is poor chlorophyll production the seedlings die before any is produced. Seedlings at the five- or six-leaf stage are usually green, and mature virescent plants are indistinguishable from normal green plants. There is also the seedling character "glossy" which is sometimes used in courses of genetics. Boxes of segregating seedlings, which all appear identical, are sprinkled with water; normal maize seedlings shed the water completely, whereas it collects and remains as drops on leaf surfaces of glossy seedlings. This character is detectable as soon as the first leaves emerge from the soil. Studies such as these help to link genetics and seedling properties for practical crop improvement.

Seedling counts sometimes give better genetic ratios for segregations than adult plant counts, especially when the adults carrying specific genes are weak types that easily succumb to environmental vicissitudes. Several genetically controlled seedling characters of this type are known in peas *(Pisum sativum)*. One, called "screwball", gives dwarf plants with curled and crinkled leaves; they are, therefore, more subject to environment than normal peas (Kellenbarger, 1952). As screwball seedlings are readily detected, ratios for this gene are better when counts are made from them rather than on adults. Furthermore, this

TABLE III

MAJOR GENES CONTROLLING CHARACTERS OF COTYLEDONS IN SEEDLINGS OF ECONOMIC CROPS

Crop	Character	Effect and Inheritance	Authors *
Gossypium spp.	Deep red (*G. neglectum*) v. green (*G. herbaceum*) cotyledonary stalk.	F_1 faint red; F_2 segregates in a non-simple fashion.	Kottur, 1923.
Glycine soja	(Green v. yellow) cotyledon colour	(a) Maternal inheritance.	Owen, 1927.
		(b) Yellow dominant to green (digenic) F_2 15 yellow : 1 green	Owen, 1927.
		(c) Green dominant to yellow	Terao and Nakatomi, 1927.
Lens esculentum	(Orange v. yellow) cotyledon colour	Orange dominant to yellow	Tschermak, 1890.
Phaseolus vulgaris	Cotyledon colour	Yellow dominant to green	Tschermak, 1919.
Pisum	Cotyledon colour	Yellow dominant to green (sometimes complicated)	Mendel et al.
	Cotyledons' form in seed	Smooth dominant to wrinkled	
Datura stramonium	Cotyledon habit	Normal flat type dominant to curled	Blakeslee, Morrison and Avery, 1927.

* Full references are given in Matsuura (1933).

character is also useful in studying linkage relations, as for linkage of "screwball" with yellow-green cotyledon colour: F_2 phenotypes occur among seeds produced on F_1 plants, as cotyledon colours are determined by genetic constitution of the embryos. The F_3 data are obtainable without growing the F_3 generation to maturity in studies of coupling and repulsion. Seedling genes such as these are also helpful in biometrical genetics for estimating the number of effective factors. Table III provides a list of major gene-controlled cotyledon characters of some economic crops.

Sometimes genetic ratios are obtainable only at the seedling stage; for example, Kellenbarger (1952) found that yellow-green pea seedlings are uniformly yellowish-green on emergence but become greener as they grow older. Hence, while seedlings are easily distinguishable, older plants are more difficult to separate. Seedlings showing the yellow-green-2 gene are green for the first week, then change to yellow-green, with some parts fully yellow; eventually the plants become greener. For accurate scoring there is, therefore, a crucial time when counts have to be made.

There are several other aspects of seedling morphology known to geneticists. For instance, several genes control cotyledon size in *Nigella damascena,* and there is some correlation of this with seed weight; generally in this species inheritance of these characters is matroclinous (Toxopeus, 1927). There is also the genetical control of the mode of germination, as in *Phaseolus,* where the cotyledons of *Ph. vulgaris* remain hypogeal during germination but in *Ph. multiflorus* remain epigeal (lit. in Matsuura, 1933). Cotyledons of the F_1 species-cross are nearly hypogeal; those of the F_2 show a continuous segregation. The F_1 plants have only half-length hypocotyls but with increased epicotyl length compared with *Ph. vulgaris,* which has epigeal cotyledons and dwarf plants; long hypocotyl is incompletely dominant to short hypocotyl. Incidentally, as a characteristic for inclusive herbaria, Anderson (1951) has advised making notes and measurements on how much *Phaseolus* cotyledons remain above or below soil level after germination. Lamprecht (1944) has found that *Ph. vulgaris* and *multiflorus* are separated only by two characters, i.e., by position of the cotyledons and form of the stigmas.

Perennial species take so long to grow, and involve so much space in experimental plots, that it is also important for a breeder of perennials to know whether his crossing technique has been accurate. *Paeonia*

species and hybrids, for example, germinate slowly and take several years to mature, but Saunders and Stebbins (1938) found a seedling character useful for checking some of their cross-pollinations. *P. tenuifolia* differs from other species of the genus, except *P. Brownii,* in having cotyledons of young seedlings regularly raised above the ground, where they become green and functional. They are retained in the seed as storage organs in other species, with occasional exceptions, and the first visible seedling leaf develops from the plumule. With *P. tenuifolia* as a parent, the F_1 seedlings had raised cotyledons as this is a dominant character.

Seedling characters can occasionally explain differences in genetic ratios. For example, Davis (1921) demonstrated differences in segregation of F_1 reciprocal crosses between *Oenothera brevistylis* and *Oe. lamarckiana,* and attributed lower ratios of *Oe. brevistylis* after malnutrition of seed to many abortive seedlings called "stumpy", in which a root fails to develop from the hypocotyl tip. Hence seedling morphology may be important in estimating the value of good seed.

Viability of seedlings has been widely used in genetical studies. An especially interesting example (which might be more widely occurring than realized when breeding some hybrid plants) is the study of interspecific hybrids of *Crepis capillaris* and *Crepis tectorum* seedlings at the cotyledon stage, i.e., whether they die or not after this stage, by Hollingshead (1930). She demonstrated a lethal factor in the heterozygous condition in *C. tectorum.* It is in radiation studies that changes in seedling viability and in morphology are, however, becoming increasingly important. Goodspeed and Avery (1933) were able in *Nicotiana tabacum* to study inheritance of an X-ray-derived mutant called "deformed", which has various phenotypical manifestations at all stages of growth. It is readily recognized in young seedlings, and so picked out at the first or second leaf stage for use in genetical study. Anderson et al. (1949) studied the effects of atomic bomb radiations on maize seedlings of the exposed generation from treated seeds, and could compare amount of seedling mottling and streaking of early leaves of seedlings with X-ray effects. The mottling and streaking changes with order of leaf appearance, first leaves being more heavily affected than later ones. Here again it is necessary to study effects on very young seedlings to obtain a true picture of what happens. The number of leaves for satisfactory examination depends on how many leaf primordia there are at time of radia-

tion, and partly on the gradual elimination of abnormal tissue. Seedlings also show reduction in vigour as a result of treatment.

A useful experimental species recently reported by Haccius and Reinholz (1953) is *Eranthis hiemalis*. Seeds can be irradiated before cotyledon development has begun, so that cotyledon abnormalities, such as polycotyly (the occurrence of extra cotyledons), are a measure of the influence of irradiation; and similarly it is possible to study the effects of treating the seeds with growth substances by the amount of tricotyly so produced. This, and similar types of species, clearly have a great future where seedling morphology is being used for testing the actions of irradiations, and of mutagenic and growth substances as to their possible use in plant breeding.

Seedling morphology has also been used in the study of facultative apomixis in *Citrus*, where it has been desirable to compare the effects of using a particular type of pollen on various types of females. Thus Soost and Cameron (1954) found that the proportion of hybrid seedlings in *Citrus* progenies is predominantly dependent on the seed-parent variety. By using trifoliate orange pollen they could accurately detect the two types, sexual and apomictic, in young seedling populations: the F_1 hybrids with trifoliate pollen had the three-leaflet character of this species, which is dominant to the single leaflet condition of common varieties. They showed in this way that apomixis is complete or manifests various degrees of facultative apomixis, or there is complete sexuality. Such information is of use where apomictic seed formation is used in the production of rootstocks, and indicates where isolation from cross-pollination by other varieties may be advisable.

In studies to determine the frequency of sexuality in polyploid facultative apomictic Rubi, Haskell (1960) has used as the female parent *Rubus laciniatus*, whose seedlings soon show the "cut-leaf" condition on their first few leaves. The true hybrids are immediately detected by their whole leaves in crosses with sexual species. By raising very large families, larger numbers of hybrid seedlings can be selected for further study than hitherto possible. The association between the frequency with which sexuals occur and the taxonomic relationship of the two parents can then be determined.

Seedling morphology may have a contribution to make in the study of heterosis in adult plants; for example, Clarke (1955) has classified cultivated varieties of tomatoes into three groups based on differences

in the first two seedling-leaves. Whether Clarke's groups can form the basis for further studies of heterosis in the cultivated tomato remains to be determined.

SEEDLINGS IN CYTOPLASMIC STUDIES

Seedling morphology as a breeding technique has unique value in particular plant selections involving cytoplasmic conditions. This is illustrated by the disease called "June yellows" of strawberries *(Fragaria* spp.), which appears at its earliest stage on the first true leaves of seedlings. The condition is very serious in some varieties of cultivated strawberries in Britain and the U.S.A.; it is due neither to a nutritional effect nor to infection, but occurs suddenly in some varieties.

Williams (1955) gives details of using strawberry seedlings to demonstrate its mode of inheritance, and as a breeding technique for selecting away from lines contaminated with June yellows. Seedlings may be green, suspiciously pale, mottled or show bad variegation. Those raised from seeds sown in February in England can be scored several times to the middle of April, the seeds being germinated on damp filter paper at 20°C and seedlings pricked out as they germinate. Symptoms of June yellows show best on leaves just fully expanded, but are less distinct on older leaves; hence several scorings are necessary if no leaves on a seedling are at a suitable stage, and as seedlings become yellow at different ages. The number of yellowed seedlings from selfing is proportional to the severity of parental symptoms in the first year. Curiously, the cotyledons always remain completely green, even though earliest symptoms of June yellows are seen in the first true leaf of seedlings.

The change to yellows is under genetical control, and onset of the "mutation" in a clone may not be for several years after a seedling has germinated. Seedlings in families with high likelihood of becoming affected usually show symptoms earlier than those in families less likely to succumb. Future selection and breeding of new strains of strawberries, at least in Great Britain, may have to be based on families with low incidence of yellows amongst young seedlings, as this indicates where there will be less trouble later on.

Numerous investigations using seedlings have been made on variegation, another cytoplasmic phenomenon. In *Salpiglossis,* for example, Dale and Rees-Leonard (1939) have analysed plastid variegation and

followed changes in pattern of variegation from the first seedling leaves to those of adult plants. Although the change is from green to colourless, seedling leaves may show single green spots on relatively large colourless areas, suggesting a reverse process. Time of first appearance of variegation is somewhat variable, and might start with the cotyledons or any of the successive seedling leaves. This variegation is believed to be determined by a recessive factor. Another example is that of Imai (1934) who studied variation in rate of recurring plastid mutations of *Hordeum vulgare* by sowing at different times. A recessive gene stimulates plastids to alter from green to white; variegated plants breed true but produce some albinotic seedlings that die in a few weeks after germination. The gene is readily detectable in the seedling stage, and albino frequency varies with time of sowing, probably being a temperature effect. As temperatures at which seedlings are raised may have various consequences on their morphology, there ought to be further exploration of using critical temperature values in seedling selection.

One of these temperature effects on seedling morphology is that controlling production of rogues in tomatoes. Rogues are off-type plants with abnormal, yet stable, forms of growth. They occur in British varieties which are highly inbred, but are absent from the more open-pollinated outdoor American varieties. Half-rogues, intermediate between normal and true rogues, are also known. Frequency of rogues is increased during germination by high temperatures (23-30°C), low light intensity and short days. The inflorescence producing the seed, and genetic constitution of the line are also important. From this evidence Lewis (1953) has suggested there is a "normal" plasmagene constitution in a rogue-free line, which is absent from a rogue-producing line. As rogues may upset a breeding programme, or their presence interfere with carefully designed statistical trials, it is useful to be able to eliminate them at a very early stage. In both kinds the cotyledons are of equal length. Seedlings of future normal adult plants have, however, the first two leaves of unequal length, whereas seedlings which develop into rogue plants have the first two true leaves nearly equal. Hence seedlings of the two types are readily separable.

Hill (1925) has found reciprocal differences in cotyledon form and size in reciprocal hybrids between *Digitalis purpurea* and *D. lutea*. The cotyledons of the hybrids resemble these of the maternal species, but the true leaves of these hybrids are alike in the reciprocal crosses.

Whether this difference is a mechanical one due to compression by the seed coats or cytoplasmic has still to be determined, but there is the likelihood of maternal inheritance.

SEEDLING MORPHOLOGY AND CHANGES OF PLOIDY

It is relatively easy to recognize seedling types which do not involve changes in chromosome numbers, such as albinos and semi-albinos. Yet early morphological recognition of other curiosities, e.g., plants with aberrant chromosomal constitution, may aid the cytologist or help the plant breeder in his search for new forms. Identification of haploid and polyploid plants early in a breeding programme, or following the use of colchicine, can be of great use to the plant breeder, and some examples involving the use of seedling morphology will therefore be considered here. At the same time, some attention must be paid to aneuploids, which involve losses or additions to the normal chromosomal complement, as these sometimes produce unusual forms with economic potentialities.

In his early work on tetraploid and haploid maize, Randolph (1932) studied size and distribution of stomata in the seedling stage, using a 20 x hand lens. The stomata of haploids were definitely smaller and closer together than those of diploids, while they were correspondingly larger and more widely separated in triploids, tetraploids and octoploids. Randolph and Fischer (1939) used tetraploid maize stocks in which genes for recessive endosperm and seedling characters were contributed by the seed parent, and dominant alleles came from the pollen parent. Hence parthenogenetic maternal individuals arising pseudogamously in these crosses would show recessive maternal characters. This meant that maternal diploids lacked anthocyanin colour as seedlings and could be further crossed with tester stocks; their stomata were also reduced in size. Bishop (1953) found, while searching for $4n$ seedlings among $2n$ apples, that, although leaf thickness and stomatal length are most reliable morphological tests, the polyploid seedlings characteristically have a spreading habit. According to Nilsson-Ehle (1938), tetraploid apples should be searched for among seedlings with particularly vigorous growth and with more or less rounded leaves; there are also differences in the leaf bases.

Twin seedlings are known in many genera, and they have been used on several occasions for obtaining diverse types of chromosome numbers,

including haploids, various polyploids and aneuploids. Müntzing (1937) examined twins in many species, especially cereals, and found that, whereas some twins have equal vigour, there is sometimes a difference in vigour between them. The weak dwarf seedlings, once separated, may ultimately develop into quite vigorous plants, yet these are often the ones with deviating chromosome numbers; new triploid seedlings are weaker than the normal diploids. Species with heteroploid twin seedlings include *Triticum vulgare* (2*x*/3*x*), *Tr. turgidum* (4*x*/6*x*), *Secale cereale* (2*x*/3*x*), *Avena sativa* (6*x*/9*x*), *Phleum pratense* (3*x*/6*x*/9*x* with the 3*x* representing a haploid), *Poa pratensis* and *Lolium perenne*, where deviating twin plants belonged to the same twin pair. There are complications in *Poa pratensis* from its being apomictic and very polymorphic. This method may, therefore, offer a means of breeding and selecting within apomictic populations which throw heteroploid twin seedlings. Generally, where there are differences in seedling morphology, about 75% of the weaker seedlings of the pair have chromosome numbers deviating from normal.

A useful table of these and other species is given by Müntzing (1938) and includes a list of observed types of deviations; approximately 4% had deviating chromosome numbers in 2201 twin pairs of all species. Only those twin-pairs which differed morphologically were examined cytologically. Haploids alone could be identified morphologically with relative ease, being conspicuous by their small size; triploids could not be recognized with certainty. In 16 species, there was a ratio of 77 3*n* : 11*n* : 2 4*n* out of the 2201 twin-pairs. Thus the twin seedling technique can have some value in breeding, especially where triploids can be used. For example, in apples, Bergstrom (1938) studied 16 seedling pairs from triploid Blenheim Orange and five pairs from triploid Belle de Boskoop, examining cytologically the so-called *a* and *b* members, some of which died. All were diploids ($2n = 34$) except one smaller plant with $2n = 51$, i.e., triploid; unfortunately no tetraploid seedling was found. In commercial fields of Sea Island Cotton recognizable haploids occur at 1 : 3-4000 plants (Harland, 1939), and these are due to frequent occurrence of seeds with two embryos, one being a haploid and one being a normal diploid.

Monoploids, which are haploid (n) plants in the sporophytic phase, are detectable in maize, for example, at the seedling stage, not only by using marker genes, but the young plants resemble their seed parents, being mostly of maternal origin (Chase, 1952). Those having the first

leaf as long as the equivalent one of the seed parent are usually diploid. These are discarded and further screening of remaining seedlings is cytological, from chromosome number counts of their root tips. Haploid plants are usually smaller than corresponding diploids, with leaves thinner, narrower and less coarse than those of diploids. Haploids have smaller cells, and usually have a shorter vegetative period than diploids. There are several metrical differences: the stomatal ratio, for example, in *Nicotiana rustica* of n : $2n$ is 26.4 to 46.7 microns, i.e., 1/14, and root cells of haploids are smaller than those of diploids. Occasionally exceptional n plants occur which are the same size as $2n$ plants. Haploidy may result from species crossing. One example is that of Gates (1929) who produced a haploid in *Oenothera* from crossing *Oe. rubicalyx* with *Oe. eriensis*. The hybrid seedlings were yellowish, developing little chlorophyll and died when the stored nourishment was exhausted, but a surviving seedling had the haploid chromosome number and possessed the red pigment of *Oe. rubicalyx*. It appears to have developed parthenogenetically. The reciprocal cross produced only normal diploid hybrid seedlings.

Whether spontaneous polyploid plants can generally be isolated in natural diploid populations at the seedling level cannot at present be answered. It is known that polyploid seedlings of some species do not necessarily differ morphologically from normal diploids of the stock which produced them. Navashin (1925), for instance, found a triploid and a pentaploid mutant in 150 seedlings of *Crepis capillaris* but could recognize them only cytologically. On the other hand, there are two main chromosome-races of *Ranunculus ficaria* in Britain, a diploid and a tetraploid only recently recognized; these generally can be separated from the first leaf stage onwards in plantlets arising from root-tubers (stem-tubers occurring only in the tetraploid race). The diploids generally have on their leaves a mottling effect consisting of dark anthocyanin and light green patches, which is absent from, or only faintly present in tetraploids (Marsden-Jones and Turrill, 1952). I have found in winter that pots containing the tetraploid strains can be separated from those with diploids, as the $4n$ stem-tubers lie on the surface of the pots but are absent from those containing diploids.

Seeds and seedlings have both been widely used in the colchicine technique for producing polyploids, and morphological differences play an important part in identification of seedlings successfully made polyploid. Species differ widely in their response to treatment, some species

readily responding to colchicine treatment. Diploid seedlings of *Cosmos,* for instance, are so sensitive to the drug that it is necessary merely to moisten soil over and around the seedlings with an aqueous solution of less than 0.1% after germination before the seedlings have emerged (Hayes and Immer, 1940). Hypocotyl length of germinating seedlings has been used by Douwes (1952) to study the effect 24 days or more later after various colchicine treatments. Generally there is a decrease in length with dosage, but the crucial test of whether there has been successful induction of polyploidy is by chromosome counts in pollen mother cells and by stomatal length measurements. New polyploids induced by colchicine may have greater stomata size; yet a plant may be tetraploid without showing change.

Krythe and Wellensiek (1942), in a survey of the morphological action of colchicine, state that cotyledons of colchicine-treated seeds are unaltered in shape as a result of induced polyploidy. But there are differences between the cotyledons of diploid and autopolyploid strains of the same species. For instance, cotyledons of a diploid strain of radish (Sparkler variety) and of a colchicine-induced autotetraploid strain (Scarlet Globe) have been examined. Photographic silhouettes of the cotyledons fully expanded (Fig. 1) reveal that tetraploids have larger cotyledons than diploids and are easily recognizable by this. Tanigawa and Churo (1953) state, too, that cotyledons of colchicine-induced tetraploids of *Lobelia inflata* are larger than those of diploids; likewise, leaves of the tetraploids are thicker and darker, and more rounded. Clearly, then, size and perhaps even shape of cotyledons can be important for discriminating between diploid and polyploid populations and plants of some species. First leaves of seedlings after colchicine treatment are mostly irregularly lobed or of crisped form; also they may be coarser and with more hairs than the controls. The leaves are dark green or may often show dark and light green areas; this indicates a $2n$ - $4n$ mixture in the tissues. Generally, increased chromosome number goes with darker green leaves from an increase in chlorophyll content.

Blakeslee (1921) believed that the first so-called "mutant" that he discovered in *Datura stramonium,* known as "globe", may have been because of the ease with which it is distinguishable from normal plants. It differs in capsule shape, types of spines on the capsules and arrangement of its broad leaves in the adult stage. Blakeslee pointed out, too, that it can be recognized at any stage of development: indeed, it is the

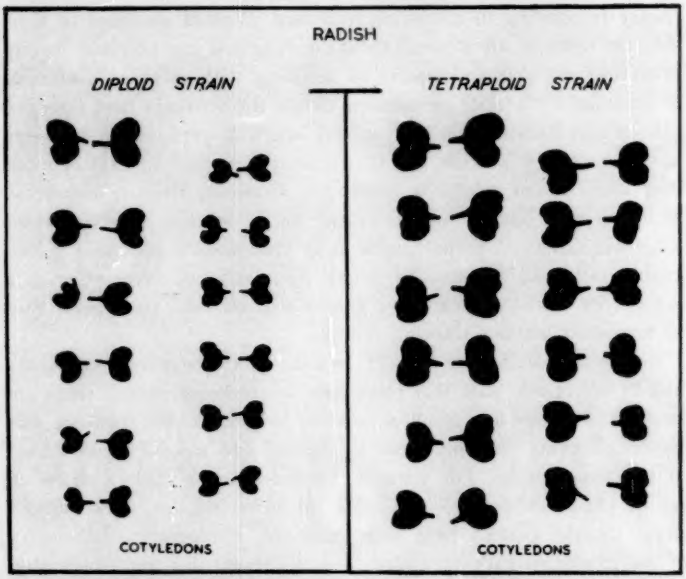

Fig. 1. Silhouettes of cotyledons from seedlings in a diploid strain (left) and a colchicine-induced autotetraploid strain (right) of radish *(Raphanus sativus)*.

only mutant he found in this species that could readily be identified in the seed pan. The first leaves are shorter and broader at the cotyledon stage, and it was thus possible to grow it in large numbers for genetic studies of its mode of inheritance. Actually "globe" is a trisomic form.

Rather than attempting a wide survey of the morphological features of aneuploids, I shall confine attention to their occurrence and identification from seedlings in tomato *(Lycopersicum esculentum)*. Primary trisomic plants have in the diploid stage one extra chromosome, representative of any member of the normal complement. Hence in tomato, where $2n = 24$, twelve types of trisomic plants are possible: eleven of these have now been identified morphologically and correlated with the various chromosomes which themselves are distinguishable at pachytene (Rick and Barton, 1954). Studies of this kind are helpful in identifying a given linkage group with its respective chromosome, and so ultimately useful to the plant breeder. Tomato trisomes show differences in morphology at the seedling stage: thus all trisomic seedlings are much slower growing and paler in colour, except possibly triplo-3

and -12. They are, therefore, readily distinguished at a very early stage. Rick and Barton (1954) have reported that trisomic seedlings of several types are readily distinguished from sister diploid seedlings, e.g., triplo-5 seedlings remain very much smaller and more compact; triplo-8 have leaves with glossy convex surfaces at a very early stage. Triplo-9 seedlings are like -8 types, but half may lose their meristems and cease to grow after the first or second leaves have appeared, or may not advance beyond the cotyledon stage. Trisomic seedlings are generally weaker and slower to emerge than normal diploid seedlings.

The technique of using seedling morphology in the detection of polyhaploids in marrow-stem kale *(Brassica oleracea* L. var. *acephala* D. C.*)* has been applied by Thompson (1956). Non-hairy first-seedling-leaf is recessive to hairy, and five plants which were non-hairy were pollinated in the bud stage by a single plant homozygous for hairy. Four non-hairy plants were found among 2674 seedlings, three having sterile pollen; two of these had smaller flowers than the normal diploids and relatively smaller stomata. Their pollen mother cells revealed them as haploids. These can be used as the basis for re-synthesising homozygous diploids.

ECONOMIC APPLICATIONS OF SEEDLING MORPHOLOGY

Some examples may now be given of seedling morphology and other seedling techniques which have applications in modern horticultural and agricultural practices directly and indirectly associated with applied genetics. For instance, in double-flowering stocks *(Matthiola incana),* one type called 'Slender' has been reported by Frost and Lesley (1954), which is a trisomic that produces an especially high proportion of doubles. Most of its single offspring are 'Slenders' which are recognizable as young seedlings by their narrow, long petioled leaves. Very narrow leaved seedlings occasionally occur which are tetrasomics with two extra chromosomes. Most singles can, therefore, be eliminated by discarding the 'Slender' seedlings when they have several leaves. Hansen's strain of stock also uses seedling differences to separate singles and doubles; seedlings with light green cotyledons always are double, whereas those with dark green cotyledons are single. This distinction is enhanced by sowing the seed at 10-15° C and lowering the temperature to 10° C when the cotyledons are formed; the colour difference is more pronounced at lower temperatures.

The use of male-sterility in hybrid vigour has been discussed by Bateman (1953) in an up-to-date review of methods of vegetable breeding. He suggested it would be helpful if male-sterile and heterozygous plants were recognizable at the early seedling stage. Currence (1944) had already recommended using linkage between male-sterile tomato plants and apple-green hypocotyl colour, these being readily detected in seedlings even before the cotyledons have completely expanded. Since then Currence has made available male-sterile lines linked with the genes for green hypocotyl and for potato-leaf, a character which is recognizable from the first seedling leaves. Several other seedling characters are now known in tomato, a crop to which geneticists and plant breeders have directed much attention. According to Butler (1952), as many as 200 tomato plants to the square foot can be raised and classified for seedling characters. Useful genes include green v. purple stem, yellow v. green cotyledon, dwarf v. tall hypocotyl, woolly (i.e., grey leaves and stems) v. normal seedlings; these are suitable for both teaching genetics and research in developmental genetics. Characters such as these, and others for cotyledon characters like narrow, rolled, mottled and giant cotyledons described by Young and MacArthur (1947), could also be useful as marker genes in detecting hybrid tomatoes. Recently Robinson and Rick (1954) have described four new tomato seedling mutants which are not associated with any cytological abnormalities; the early growth stages show diverse abnormalities, and their cotyledons are generally smaller and paler than those of normal seedlings.

The use of other kinds of seedling techniques may now be considered. For instance, when selecting material from a nursery for establishing a plantation, as in coconuts and oil palms, it is valuable to know whether there is economic benefit from choosing apparently most vigorous seedlings. The Coconut Research Scheme in Ceylon (cf. Toovey, 1953) has found considerable benefit from ruthless seedling selection: seednuts failing to germinate within four months, and half the sprouted nuts, are rejected on transplanting to the nursery. This results in selected seedlings planted in the field starting to bear earlier and yielding more heavily than unselected seedlings.

Toovey (1953) and his colleagues have germinated oil palm *(Elais)* seedlings in raised trays; and before planting in the nursery, graded them into early germinators and late germinators; these were again sub-divided into more and least vigorous seedlings. There were sig-

nificant differences for survival and vigor corresponding to selection for vigour in the trays, but rapidity of germination did not exert much influence, although Toovey (1953) thought that best seedlings were those germinating later. Although least vigorous seedlings, including those from either later or earlier germinations, were significantly worse in vigour than the control, there were no morphological differences in biometrical characters such as numbers of leaves per palm.

There is a possibility in dioecious species of differentiating sexes from morphological differences between male and female plants, and this could possibly be extended to the seedling or early plant stage. For instance, Winge (1923) has examined frequencies of females to males in some dioecious species, and found a general preponderence of males. He thought that, at least for hemp *(Cannabis sativa)*, there was a noticeable difference in vigour at a comparatively early stage between male and female plants. D. Lewis and L. Crowe (unpublished) have studied seedlings of *Origanum vulgare* to see whether any characters are associated with female, male and hermaphrodite plants, but could not detect any differences until flower formation. On the other hand, from measuring lengths and breadths of equivalent leaves of young plants, they found that males generally have narrower leaves than females in some populations of *Melandrium album* and *M. rubrum*. There was some overlapping of measurements, so that clear-cut separation was not possible, but most plants could be classified as to their sex in this way. Such metrical differences in the sexes, however, are not always found in populations of these species.

Some cultivated species are dioecious, and sexuality often is simply inherited and associated with sex chromosomes. The sex of a particular asparagus plant, for example, cannot be absolutely determined except from the flowers, although Flory (1952) has shown there are several important quantitative differences between staminate and pistillate individuals. In asparagus *(Asparagus officinalis)* the staminate plants tend to flower first, and are shorter with more shoots in the first two years than pistillate plants. Yet there is relatively little information on general recognition of sex types from seedling morphology, although this would have great practical value in breeding experiments. There are, however, some clear-cut examples of sex determination from morphological characters which are of use in plant breeding. For instance, Crane and Lawrence (1931) found that male plants in raspberry *(Rubus idaeus)* characteristically have obtuse foliage, and this charac-

ter is detectable at the first leaf stage; Rozanova (in *loc. cit.*) found in *R. chamaemorus* that male plants have obtuse leaves but that those of females are incised with more acute apices. Hence in raspberry breeding these non-productive male plants can be detected and discarded while still in the seed-box. This character is also of use to *Rubus* ecologists, as the underground spread of a male plant through the soil can be followed without confusion from adjacent hermaphrodite plants and other germinating seedlings, as it is seedless. Spinach *(Spinacea oleracea)* plants are mainly dioecious, although monoecious individuals occur, and extreme male plants are readily recognized by their sparse foliage and small leaves, which are usually thin and sharply pointed. There are also so-called vegetative males with well developed leaves like those of monoecious and female plants (Drewes, 1943); hence only extreme males can be eliminated at the seedling stage. Some trees also have the complication of dioecism; e.g., yews *(Taxus baccata)* segregate male and female plants. Where yew walks are planted with seedlings, it is not at present possible to predict their sex, and it would be helpful if one could discard most of the non-fruiting males before planting.

Differences in germination rate and vitality of seedlings of various germinating powers may vary according to whether a crop is heterozygous or highly inbred and homozygous. Any variation among homozygotes is primarily attributable to environmental effects, such as differences in available water for the germinating seeds, and to slight unevenness in permeability of the seed-coats. Hence knowledge of the breeding system of a species is important for the plant breeder, and he must know whether it is cross-breeding, inbreeding or somewhere between these extremes, before he is able to plan his breeding campaign. Seedling morphology has already been shown to be helpful in determining the natural breeding behaviour of a wild species. Thus in three strains of groundsel *(Senecio vulgaris)* Haskell (1953*b*) found that the seedlings of each from generation to generation were characteristic. The general vigour, intensity of green pigmentation and first leaf size differed between strains, but seedlings within each strain were remarkably similar. This helped to demonstrate that natural self-pollination was usual for groundsel.

Seedling morphology has also been widely used in studies of the breeding systems of cultivated plants under field conditions. Such information is not only a useful adjunct to our knowledge of the species,

but is important when determining effective isolating distances for avoiding contamination in seed crops (Haskell, 1944). Seed appearance, seedling characteristics, and juvenile and adult plant morphology are characters which have been used in experiments for determining the amount of cross-pollination of various economic crops. Table IV lists experiments in which seedling morphology has been used; stem pigmentation, and leaf shape and colour have been most helpful, but other characters such as root shape of seedlings and glandulation of cotyledons have also been useful. When studying breeding behaviour in the field, the advantage of using seedlings is that progeny of tester plants may be grown during winter in large quantities without occupying much glasshouse space. In turnips, for instance, Bateman (1947) used contamination between "white, swollen rooted" versus "red-tops non-swollen rooted" as the discriminating characters. The F_1 population could be scored by pigmentation or non-pigmentation in young seedling leaves and at the leaf bases, juvenile and adult plants being the same in this respect. Griffiths (1950), however, using a similar type of marker in *Lolium perenne,* thought it advisable to check the non-pigmented seedlings, since anthocyanin can show up in some adults, even when undetected in the seedling. Even so, there is still considerable reduction in the number of plants that have to be grown to the adult stage.

Seed characters have also been widely used, as the xenia effect of cross-pollination in some crops is seen immediately on the kernels, provided a correct genetic background has been arranged. Thus in maize, characters like yellow v. white endosperm, and starchy v. sugary seeds can be determined on dried ears. This method, therefore, has the additional advantage of not having to sow the seeds, and observations on F_1 material are made directly while it is still on the mother plant. But the experiments have to be planned to avoid pitfalls due to phenomena like pollen competition effects (cf. East and Jones, 1920).

Seedling morphology has also been utilized by Mullison and Hummer (1949) in studying the effects of 2,4-D vapour on seeds of various field and vegetable crops. For instance, there were leaf modifications in beans, abnormal irregular swellings on pea roots and stunting of cotton seedlings; in cotton McIlrath, Ergle and Dunlap (1951) obtained some seedlings which exhibited injury from seeds in bolls initiated eight weeks before application of 2,4-D. Likewise, when studying the effect of DDT on germination of cucumber *(Cucumis sativus),* a particular

TABLE IV

SOME ECONOMIC EXAMPLES OF SIMPLY INHERITED SEEDLING CHARACTERS USED IN STUDIES OF NATURAL BREEDING SYSTEMS.

Crop cultivated	Character (Dominant characters given first)	Author
Blackberry *(Rubus)*	Glandular v. eglandular cotyledons.	Haskell, *(loc. inst.)*
Kenaf *(Hibiscus cannabinus)*	Divided v. entire leaves.	Tamargo and Jones, 1954
Upland Cotton *(Gossypium)*	Female: red v. virescent yellow leaf and Pollen: red v. green leaves.	Finkner, 1954
	Glandular v. glandless hypocotyls.	McMichael, 1954
Blue and yellow lupines *(Lupinus angustifolius* and *L. luteus)*	Purple v. green seedling pigmentation	Wallace, Hanson and Decker, 1954.
Muskmelon *(Cucumis melo)*	Green v. yellow green (recognizable at all stages).	Whitaker and Bohn, 1952
Tomato *(Lycopersicum esculentum)*	Normal v. potato leaf (seen on 1st leaves) Presence or absence of stem colour (anthocyanin) on seedlings.	Currence and Jenkins, 1942
Jute *(Corchorus* spp.*)*	Red (anthocyanin) v. green seedlings, or young plants.	Ghose and Das Gupter, 1945
Radish *(Raphanus sativus)*	Red, short round roots v. white, long tapering roots, F_1 = purple, intermediate conical shaped roots on seedlings.	Crane and Mather, 1943
Perennial ryegrass *(Lolium perenne)*	Red base v. non-red base seedlings — identified by colour of coleoptiles (non-red base seedlings checked when adult).	Griffiths, 1950
Turnip *(Brassica rapa)*	Red pigment v. white in young leaves and at leaf bases.	Bateman, 1947*a*
Beet *(Beta vulgaris)*	Anthocyanin pigmented v. non-pigmented in young seedlings, detectable even as radicle emerges from germinating seed.	Bateman, 1947*b*
Maize *(Zea mays)*	Starchy v. sugary endosperm of F_1; seedlings within caryopses on maternal parent.	Haskell, 1953*a* Bateman, 1947*b*
Green sprouting broccoli *(Brassica oleracea)*	Normal v. glossy leaved seedlings.	Moore and Anstey, 1954

date for shedding the seed-coat four days after planting was used by Hopkins and Toole (1950), or they relied on inhibition of root and hypocotyl elongation, as in squash *(Cucurbita pepo)*.

SEEDLING MORPHOLOGY AND PRACTICAL PLANT BREEDING

Time between generations is the great enemy of geneticists and plant breeders, and so of great practical value is the ability to raise two generations in the time normally taken to raise one. Methods of shortening the life-cycle are welcome, therefore, provided they do not upset the normality of the plant and interfere with the breeder's ability to select. One method for maize has been described by Mes (1953) who germinated prematurely harvested seeds; by speeding up the life-cycle she obtained two generations a year in Pretoria, South Africa. The growth rate of plants from immature seeds is often slower in the early stages, but faster in later stages than that of plants from mature seeds. The coleoptile was unable to break through the fruit and seed coats in many immature maize kernels with a water content above 70%, and it kept on growing underneath these coats and curling round the seeds inside. This behaviour can be overcome by making a slit near the tip of the coleoptile for it to grow through.

Breeding some plants takes up not only much time but also much space; this is especially true of top fruits and soft fruits. Hence any method in which seedling morphology can help to reduce space in the field is welcome. One such technique can be illustrated from breeding new thornless blackberries *(Rubus* spp.*)* from families segregating for thorns and no thorns. When M. B. Crane raised Merton Thornless blackberry, his selfed generation from the F_1 parent (John Innes blackberry) comprised 37 thornless and 835 thorned plants. This segregation represents the 1:20.8 ratio expected for a tetraploid with chromatid segregation. One selected thornless plant was backcrossed to John Innes, and a backcross generation of 11 thornless and 31 thorned plants raised in the field; the Merton Thornless plant was chosen from the former group and vegetatively propagated. Thus Crane raised 914 plants in the field, and, as in England *Rubus* is planted at six feet between rows and at two feet within the row (i.e., a plant requires 12 sq. ft), Crane's total crop occupied 914 x 12 = 10,968 sq. ft. Actually only 48 thornless plants were available, and these occupied only 48 x

12 = 576 sq. ft. Hence 10,392 sq. ft., representing 95% of the total field area, were wastage. Economically this represents a heavy financial drain, considering that cultivated *Rubus* crops are very expensive to grow, for they require wooden stakes and wires besides extra costs due to difficulties of handling them.

The first stage of Crane's experiment has now been repeated on a much larger scale, yet much less field space has been used by means of elimination based on seedling morphology. Forty-two seed boxes were sown containing a total of 15,935 seeds, i.e., an average of 380 seeds per box. There was a total germination in the first year of 12,463 seedlings, of which there were eventually 452 probable thornless and 12,011 thorned. The category of these numerous seedling plants was determined at the cotyledon stage by examining the cotyledons with a hand lens; seedlings with glands around the edges of the cotyledons later grow into thorned plants, whereas those with cotyledons having no glands around the edges normally produce thornless plants (Fig. 2). Only 15 seed-boxes were used for pricking out the 528 seedlings con-

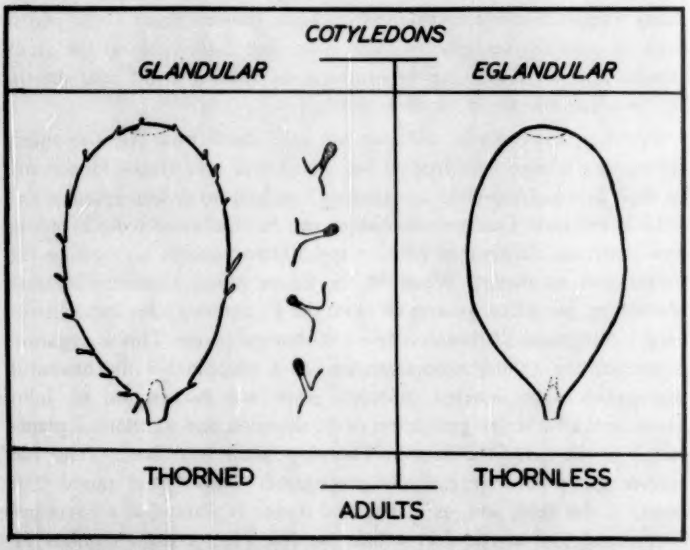

Fig. 2. Glandular and eglandular cotyledons in blackberry *(Rubus)*. These correspond to thorned and thornless adult plants. This character is controlled by a single gene, here acting in a tetraploid.

sidered to be eglandular but which included 76 misclassified glandular ones. Misclassification is more frequent among late germinators, which sometimes have difficulty in emerging from their testas; hence they often have bruised cotyledon edges where the glands would be. These 528 seedlings occupied 15 seedboxes, i.e., there was an average of 35 seedlings pricked out per box (14¾″ x 8¾″). These boxes occupy in the glasshouse a negligible area of about 15 sq. ft. Rejection of the 76 misclassified plants was possible before planting out in the field, and of the remaining 452 plants, only 396 were alive at planting times as some segregates were very weak; 10% were lethal seedlings. Field space is thus saved from use by the weakest plants.

Thus eventually 396 seedlings were planted out in the field, and these occupied 12 x 397 = 4764 sq. ft. Again it was found that there had been misclassification of 17 plants, which had a few small prickles and hence were overlooked earlier in the seed boxes. Hence loss of field space through misclassification was only 204 sq. ft. out of a total planting of 4764 sq. ft. (4%). Furthermore there is very little wastage of field space from death of lethals and unsuitable weak plants as these are eliminated while still in the seedling boxes. Thus we now have 379 thornless seedlings occupying in the field 4764 sq. ft., whereas Crane had in the equivalent experiment 37 thornless plants in 10,464 sq. ft. Saving of field space is obvious, even though the present experiment is 14 times the size of the original and contains ten times the number of thornless plants after rejecting misclassified seedlings. The result of using a seedling classification based on a single gene difference affecting cotyledon morphology is clearly seen in this experiment.

Other examples are known of the use of seedling morphology to side-step growing large numbers of plants unlikely to be useful in selection. Thus resistance in cotton to jassid attacks is attributable to the long dense hairs on the under surfaces of leaves, which if sufficiently long confer immunity. R. L. Knight (private communication) has suggested that seedlings could be preliminarily assorted into more and less resistant groups by their hairiness. This would reduce the potentially less resistant plants being grown on to the adult stage when selecting for jassid resistance. Resistance is basically due to a hairiness gene (H_1) in *Gossypium barbadense* and a number of minor hairiness genes, e.g., "hairy v. glabrous" (cf. Knight, 1952).

There is, too, in green sprouting broccoli (*Brassica oleracea* var.

italica), a mutant called "glossy" distinguishable from normal in six-weeks old seedlings. The leaf blade lacks the normal whitish bloom, which gives it a shiny dark green appearance. Such seedlings are highly susceptible to attack by cabbage flea beetle (Anstey and Moore, 1954).

It is possible, also, when breeding potatoes to discard at the seedling stage those with typical symptoms of leaf roll, which may be a genetic simulation of a virus disease. Selection in the first year can also be made on potato seedlings for tuber shape and length of stolons; seedlings with small tubers are discarded, those with a limited number of uniform size being preferred (Tedin, 1948). In crosses between cultivated potato varieties, five to ten per cent are selected for clonal reproduction for another one or two years, with further selection before commencement of trials. Hence seedling selection precedes clonal propagation in potatoes. Seedling colour is always associated with flower, stem and tuber flesh colour of adult plants in potatoes *(Solanum* spp.*)* (Müller, 1923), and the differences between blue, red and green seedlings are easily observed, the colour showing best at the upper end of the hypocotyl below the cotyledons (Sirks, 1929).

Dicotyledons normally have two cotyledons per seeding (dicotyly), but occasionally there is only one (monocotyly), or there may be three or more cotyledons (polycotyly) with intermediate types linking the series. A review of this phenomenon, called "pleiocotly", and which is polygenically controlled, can be found in Haskell (1954a); examples

TABLE V

PLEIOCOTYLY AND QUANTITATIVE SEEDLING CHARACTERS IN CULTIVATED BRASSICAS.

	Seedling Type	
Savoy Drumhead Cabbage	**Dicot**	**Tricot**
Weight in gms. of 10 seedlings (roots removed at soil level) at first leaf stage (from commercial seed)	0.975 ± 0.20	0.749 ± 0.30
Couve Tronchuda	**Dicots**	**Pleiocots**
Heights (ins.) of 8-month old plants (1st generation plants after selection for pleiocotyly)	8.92 ± 1.15	9.75 ± 1.41
Kale		
Heights (ins.) of 8-month old plants (1 generation of selection for pleiocotyly)	10.58 ± 2.22	5.0 ± 0.71

were then given from economic plant breeding, where adult plants raised from pleiocot seedlings were compared with those from dicot seedlings. Pleiocotyly selection has been especially studied in beet, sugar-beet and cultivated forms of *Brassica* (cf. Table V). This method, which permits relatively easy examination of very large populations, is here only briefly referred to, since the use of seedling pleiocotyly as a breeding technique is available in Haskell (1954b). It is possible, too, that frequency of pleiocotyly in varieties of some species might be helpful as one means of checking F_1 hybrids. Table VI gives the frequency of

TABLE VI

FREQUENCY OF PLEIOCOTYLY IN CULTIVATED TOMATOES.

Variety	Total No. of Seedlings	% Pleiocotyly
Potentate	411	0.24
Harbinger	419	1.19
Clucas 99	222	13.06
Mean for 3 commercial varieties		4.83
Leafmould Register x Vetemold	114	1.75
Leafmould Resister x dpsory	57	7.02
Potentate x E.S.1.	37	2.70
Stonor's M.P. x Potentate backcrossed 6 generations to Money Maker	36	2.78
Mean for 4 hybrids		3.56

pleiocots in three varieties and F_1 hybrids and a backcross of tomato; in this small sample frequency of pleiocotyly is too low for distinguishing them, except for Clucas 99 and one hybrid. However, the technique might apply to exceptional varieties and hybrids that happen to have high pleiocotyly. There is the additional drawback in tomato that frequency of pleiocotyly depends on which inflorescence has produced the seeds (Haskell, 1954*c*).

Polycotyly has been intensely studied in conifers for diagnostic purposes; Buchholz (1939) has recorded the frequencies of cotyledon number in the closely related big tree *(Sequoia gigantea)* and redwood *(S. sempervirens)*, and found that seedlings of the former species have three, four or five cotyledons, with a mean value of 3.7 cotyledons per seedling, whereas seedlings of the latter have only two. Butts and Buchholz (1940) have given details of the distribution of cotyledon number in numerous species of conifers and suggested their use in

diagnosis. The three largest pines in height and cone size *(Pinus coulteri,* P. *lambertiana,* P. *sabiniana)* also have the highest cotyledon number. More recently Griffith (1953) found that seeds of the same size from a single tree of *Cupressus lusitanica* produced seedlings with two to eight cotyledons, and he raised large quantities of dicots, tricots and tetracots. The tetracots at first grew slower than dicots and tricots, but tetracots and tricots showed significantly greater heights than dicots during the year after field planting.

The frequency distribution of cotyledon number in conifers is related to the species, and so indirectly to the distribution of that species. (Table VII). Hence the use of seedlings with particular cotyledon num-

TABLE VII
COTYLEDON NUMBER IN PINACEAE
(Summarized from Butts & Buchholz, 1940)

Genus	Chromosome No.		Mean Genus Cotyledon No.	Range
	x	*n*		
Cedrus	12	2	9.0	13-5
Pinus	12	2	8.1	18-3
Pseudotsuga	13	2	6.8	12-4
Picea	12	2	6.4	15-2
Abies	12	2	5.9	10-2
Larix	12	2	5.7	8-3
Pseudolarix	11	4	4.4	7-4
Keteleeria	12	2	4.2	4-2
Tsuga	12	2	3.7	7-2

ber in polycotylous species for forestry establishment might be important, by determination of which cotyledon number is the most frequent for that species; and those seedlings with the median cotyledon number might prove best for local plantations. On the other hand, where it is hoped to extend the range of a species, selecting away from the median cotyledon number may have a beneficial adaptive effect. Even so, practical results have been obtained where polycotyly has been tried out as a selection technique in tree breeding (Haskell, 1949). By sowing seeds harvested from one tree of *Laburnum vulgaris,* a monocot and tetracot as well as numerous dicot seedlings were obtained. The monocot, tetracot and a dicot seedling taken at random as control were potted and later planted out. The control seedling was vigorous, grew rapidly and produced healthy green leaves. It has branched frequently, has maintained its leaves during frosts, and has

flowered regularly for several years. In contrast, the main stem of the tetracot seedling immediately bifurcated and produced small green leaves. It did not divide again and grew only a small amount before dying, perhaps because the genic unbalance was too great for its normal survival. At first the monocot rivalled the control in vigour, but it failed to branch, and gradually the green leaves were replaced by more or less yellow leaves still able to photosynthesize, the plant being alive yet after seven years. It remained unbranched for several years, but now has a few sparse branches; it differs from the control in rapidity of leaf drop after night frosts, and it always breaks dormancy later. This monocot has flowered and has a highly ornamental appearance, owing to the attractive effect when the sun shines on its golden-yellow leaves. It could be propagated by grafting on stock of normal laburnum if it proves of sufficient interest to horticulturists.

The value of young seedling characters correlated with adult characters are at their premium in fruit and forest tree breeding, as Luther Burbank realised, for tree breeding occupies much field space. Seedling morphology as a plant breeding technique, therefore, has greatest importance in the improvement of perennial crops and those that take a long time to become adult, like forest trees. Modern tree breeding is coming more to depend on Mendelian and evolutionary principles, but even so, the recent review by Richens (1945) reveals how little such genetical characters have been used. A start in this direction has already been made by Swedish forestry workers who carefully choose seed for planting (cf. Royal Board of Private Forestry, 1950).

Seedling identification is sometimes required in forest tree breeding; e.g., in *Pinus* there has been a need for a practical method of distinguishing races at the seedling stage. Phototropic response is considered to indicate geographical origin and so is useful for diagnostic purposes. Some of the seedling characters used for selection in forest tree breeding, as in *Pinus* and *Picea,* are fresh and dry seedling weights; nitrogen and ash of one-year old seedlings; seedling size; ratio of hypocotyl length (with or without radical length) to cotyledon length; and the ratio of root to shoot. These values are characteristic of latitudinal topoclines, but single genes manifesting at the seedling stage are rare. There is clear-cut monofactorial inheritance of pigmented and green seedlings in copper beeches *(Fagus sylvatica);* only the former type with anthocyanin are desirable for breeding copper beeches, and they are readily identified at an early stage.

Gourley and Havis (1935) have recommended further use of plant anatomy in agricultural research and have shown how anatomy, e.g., in radish, is related to growing conditions. Similarly one can extend the idea to sample anatomical analyses of seedling populations, e.g., in radish strains, to determine whether one strain is more likely to be succulent and another the more "woody" type. Likewise seedling morphology and anatomy in fruit tree research might offer useful economic fields of study, besides being important to plant breeders generally. They are particularly seen in the use of anatomy of seedling apples *(Malus pumila)* to determine their potentiality as dwarf or vigorous rootstocks. Beakbane and Thompson (1945) have found that rootstocks which are dwarfing and with youthful cropping have a high proportion of living cells in their roots. A rootstock is likely to have a highly dwarfing effect on the scion if it has relatively thick bark and much living tissue in the wood, whereas intermediate combinations are correlated with intermediate vigour and cropping. This is, therefore, a short-cut for assorting new seedlings before they are field tested, an especial advantage as large fruit trees do not bear fruit for ten to 15 years, and dwarf trees begin to crop only in their third or fourth year. Hence in perennial plant breeding experiments like this, early reduction in number of seedlings for testing saves field space over several years.

Beakbane (1953) has since shown good regression between "areas of rootstock bark as a per cent of the whole root" when plotted against the "logarithm of trunk girth of the scion". There is also good regression of "living tissue per cent of whole root" stocks if plotted against the "logarithm of the trunk girth of the scion." if these characters are known, prediction of future behaviour of rootstock seedlings is good. Furthermore there is a relationship in one-year old plums *(Prunus domestica)* between the ease of vegetative propagation and tissue structure outside the secondary phloem in stems. Seedling vigour can also have practical importance in vegetatively propagated varieties, as it may indicate the vigour of clones after budding. Thus Romberg and Smith (1946) found in pecan *(Carya illinoensis)* that less vigorous seedlings came from selfings, and their grafted buds were weaker on the same rootstocks than those from crossed seedlings.

CONCLUSIONS

Seedling morphology, when used cautiously, has future possibilities as a technique in applied genetics. Any short-cut reliable breeding method for a particular crop must, however, ultimately depend for its use on the knowledge and experience of the species that the breeder has. He must be in a position to know, for instance, whether, if he takes a particular class of seedlings, he might lose characters which he desires, yet for which he is not at the time specifically breeding. Thus sugar cane growers in the West Indies have found that they could be discarding the more desirable plants with higher sugar content if they established plantations only from the more vigorous seedlings. Indeed, as correlations of undesirable characters always tend to be associated with any forms of lob-sided selection (cf. Haskell, 1954*b*), precautions should be taken that these do not upset the breeding programme, i.e., by maintaining an all-round balance. Such checks would include, at least in the preliminary stages, growing to maturity those seedlings which would otherwise be discarded on a morphological basis and later comparing them with plants from the selected class of seedlings.

The present technique seems to have greatest potentiality in the breeding of polyploid crops, where the genetic segregations are somewhat more involved than for diploids. Large populations of these polyploids have to be grown to extract a reasonable number of the recessives that are wanted, when it is these that may possess desirable qualities associated with a major gene difference, as demonstrated for thornless blackberries. Seedling characters also have a useful future in tree breeding; thus Miss E. Welkerling (unpublished) has recently found that the colour of the glands on the edges of young leaves of cherries are correlated with fruit colour, so that preliminary assortment for black and white fruits can be made long before the young trees are actually in fruit. Likewise she has found it possible to tell the fruit colour of new nectarine seedlings from the colour of the barks of young trees.

Unless the breeder already has studied his crop, he may overlook the possibility of there being a lack of some correlations which he would expect from knowledge of other crops. Generally, as Luther Burbank recognised, the coloured stems of young plants indicate coloured flowers, and there is usually a correlation with intensity of pigmentation. Yet in *Primula sinensis* there are genes for coloured stems and leaves,

i.e., with anthocyanin, but the flowers remain white, as expected on seedlings with green stems. Similarly in cherries, plums and other fruits, the white or occasionally flushed flowers remain remarkably constant in spite of a range in colour of their fruits.

It is clear, then, that where seedling morphology is to be used as a plant breeding technique, it offers considerable scope, provided the breeder is aware of its limitations and if, for the particular crop where he is using it, he is prepared to accept the pitfalls in which it may involve him.

ADDENDUM

In Brussels sprout breeding at this Institute, Dr. C. North is now using the "shiny leaf" character which is readily recognizable in the early seedling stage. The differences are probably due to the absence of wax on the leaf and stem surface. This character is being used as a genetic marker in recognizing undesirable pollinations during the preparation of F_1 hybrid lines.

A rapid method for counting or measuring stomata, when screening seedlings for haploids or polyploids, has recently been described by Sarvella et al. (1961) for cotton. Scotch tape is pressed against the lower surface of the cotyledons and then transferred to a slide; no coverslip is required. This impression method is considered suitable for plants with essentially glabrous leaves. Not all material is, however, suitable for this technique.

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DIFFERENTIAL GROWTH-INHIBITORS PRODUCED BY PLANTS [1]

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[1] In most cases common and scientific names are given in this article. In some instances terminology has changed since a paper was published, or common names in another country do not correspond exactly to common names in the United States. In these cases the terminology used by the original author is given in order to avoid error.

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INTRODUCTION

In 1832 De Candolle (28) first raised the question of the existence of natural growth-inhibitors secreted by plants that inhibit the growth of other plants. He believed that such substances exist and that they are important factors in plant ecology. His theories were based largely on field observations. Subsequently, Liebig (49) for a time also considered growth-inhibitors to be important factors in plant competition. However, finding that the availability of trace elements was important in plant growth, Liebig veered to the view that the phenomena attributed to plant growth-inhibitors were really caused by variations in the supply of trace elements. His authority carried a great deal of weight, and for the next half-century, there was little interest in inhibitors.

In 1909 Schreiner and his associates (66–69) reopened the question of growth-inhibitors through their studies of infertile soils. In a series of papers they reported finding a variety of growth-inhibitors of plant origin in infertile soils, and showed that these materials, when isolated and purified, possessed important growth-inhibitory properties. Their views were not, however, widely accepted. Indeed, in a review as late as 1937, Loehwing (50) concluded that the existence of naturally occurring differential plant growth-inhibitors had not yet been proven.

A series of decisive studies was then carried out by Bonner and his associates (12, 14, 38, 39), and in his review (15) Bonner was able to show conclusively that plant growth-inhibitors are important factors in the interactions of some plants.

Subsequently, interest in this area has increased, and several aspects of the problem have been more fully explored. It now appears that plant growth-inhibitors may be more widespread and more important than was first suspected. In addition, there has been increasing interest in these inhibitors from the zoological and medical viewpoints.

The review by Bonner was concerned mainly with establishing the existence of differential growth-inhibitors. Other aspects of differential growth inhibition did not, however, fall within the purview of his article. Furthermore, in the intervening decade there have been further studies and new approaches to this topic.

This article will consider those aspects of differential growth inhibition which were not previously reviewed. The present knowledge will be evaluated, and attention called to those gaps in our knowledge which require further study.

DISTRIBUTION IN PLANT FAMILIES

Plant growth-inhibitors are widespread throughout the Plant Kingdom. Apparently, there are no limitations to particular classes or even phyla. Fungi and bacteria elaborate inhibitors of varying degrees of potency and specificity, some of which are in common medical use as antibiotics. Representatives of grasses (1, 5, 16, 30), trees (15, 18, 46, 60), shrubs (14, 78, 81) and other groups have also been shown to produce these substances. Our information is more complete concerning those plants which stand out because of a special habitat (desert plants) or because of agricultural interest. In Tables I and II are listed the plants which have been reported to secrete growth-inhibitory chemicals.

Within a particular genus there may be considerable differences in the ability to secrete growth-inhibitors. For example, the eastern black walnut *(Juglans nigra)* elaborates a potent inhibitor (18, 21, 51, 65, 70), while its close relatives, the Persian (English) walnut *(J. regia)* and the California walnuts *(J. hindsii* and *J. californica)*, apparently do not (65, 70) secrete one with the same spectrum of activity.

SOURCES OF GROWTH-INHIBITORS

Most of the growth-inhibitors studied thus far are elaborated by roots. However, in some cases other parts of a plant are the main sources of these substances. This may be a special adaptation to a particular habitat. In *Encelia farinosa,* a desert shrub, the inhibitor is found primarily in the leaves (15, 38, 39). The leaves, falling to the ground around the plant, decompose, releasing into the soil a long-acting inhibitor, 3-acetyl-6-methoxybenzaldehyde (15), which prevents other plants from gaining a foothold. *Encelia* roots are relatively deep, and if the inhibitor were secreted from them, it would probably be ineffectual against plants starting to grow on the surface. *Artemisia absinthium* (13) also secretes a growth-inhibitor through its leaves.

In some plants the inhibitor is mainly in the fruit. A good example is mountain ash *(Sorbus aucuparius)* (23). Its berries contain parasorbic acid, a differential growth-inhibitor with some interesting properties. Presumably, the presence of this chemical in the berry is an advantage in that it provides for the seedling a small area of soil in which certain other plants cannot grow until the parasorbic acid is destroyed or leached out. Thus the mountain-ash seedling is, in effect, given a "head-start" over seedlings of competing species.

TABLE I

PLANTS REPORTED TO PRODUCE DIFFERENTIAL GROWTH-INHIBITORS OF KNOWN COMPOSITION

Plant Sources	Chemical Constitution	Plants Inhibited	Parts Inhibited	Plants Resistant	Comments	References
Tonka bean *(Dipteryx odorata and D. oppositifolia)*, sweet clover *(Melilotus)*	coumarin	carrot, onion, lily, alfalfa	roots	cress, cabbage		2, 23, 64
Spreading pasque-flower *(Anemone pulsatilla)*, buttercup *(Ranunculus)*	protoanemonin	cress, corn				2, 31, 37
Thamnosma montana, Angelica glabra (58)	byakangelicin	tomato				12
Thamnosma montana	isopimpinellin	tomato				12
Thamnosma montana	$C_{16}H_{15}O_5(OCH_3)$ with isobergaptene nucleus	tomato				12
Guayule *(Parthenium)*	transcinnamic acid	guayule, pea		tomato	homologous	14, 78
Encelia farinosa	3-acetyl-6 methoxybenzaldehyde	tomato, pepper, corn		barley, oats, sunflower	inhibitor in leaves	15, 38,39
Mountain ash berries *(Sorbus aucuparius)*, wheat middlings, orange rind	parasorbic acid	onion, tomato	roots			20,23
Vanilla bean	vanillin	wheat seedlings	roots			66
Bergenia crassifolia Pyrus communis	arbutin	wheat seedlings			inhibitor in leaves	66
Oats *(Avena)*	scopoletin	oats			homologous	36, 61
Not listed	umbelliferone	red kidney bean, cucumber seedlings	roots	peas, corn, wheat		40

TABLE II

PLANTS REPORTED TO PRODUCE DIFFERENTIAL GROWTH-INHIBITORS OF UNKNOWN COMPOSITION

Plant Sources	Plants Inhibited	Parts Inhibited	Plants Resistant	Remarks	References
Clover *(Trifolium)*	Kentucky bluegrass *(Poa pratensis)*		bromegrass *(Bromus inermis)* timothy *(Phleum pratense)*		1
Kentucky bluegrass *(Poa pratensis)*	Canada bluegrass *(Poa compressa)*	foliage & roots			1
Redtop *(Agrostis alba)*	timothy *(Phleum pratense)*	foliage & roots			1
Redtop	Kentucky bluegrass *(Poa pratensis)*	foliage			1, 30
Brome grass *(Bromus inermis)*	brome grass seedlings			homologous	11
Mesquite *(Prosopis juliflora)*	tomato				12
Greasewood *(Sarcobatus vermiculatus)*	tomato				12
Viguiera reticulata	tomato				12
Creosote bush *(Larrea tridentata)*	tomato				12
Encelia frutescens	tomato				12, 57
White Bur-sage *(Franceria dumosa)*	tomato				12, 56, 57
Wormwood *(Artemisia absinthium)*	sage *(Salvia schlarea)*			inhibitor in leaves may be absinthin	13
Artemisia absinthium	groundsel *(Senecio)*		*Datura, Stellaria*		34
Peach	peach			homologous	15, 62

TABLE II (cont.)

PLANTS REPORTED TO PRODUCE DIFFERENTIAL GROWTH-INHIBITORS OF UNKNOWN COMPOSITION

Plant Sources	Plants Inhibited	Parts Inhibited	Plants Resistant	Remarks	References
Sorghum	other grasses				16
Black walnut *(Juglans nigra)*	broomsedge *(A. virginicus)*		Kentucky bluegrass	inhibitor may be juglone (27)	18, 51 65, 70
	poverty grass *(D. spicata)*		timothy		
	blackberry (*R.* spp.)		black raspberry		
	dock		redtop		
	common cinquefoil		fleabane *(Erigeron)*		
	red pine *(P. resinosa)*		ferns		
	white pine (*P. strobus*)		asters		
	apple		goldenrods		
	potatoes (21)		mints		
	alfalfa	leaves (21)	violets		
	tomatoes (21)		wild grape, clovers, Virginia creeper		
	hydrangea		thistle, ironweed		
	lilac		tall oat grass,		
	chrysanthemum		orchard grass,		
	asparagus		meadow fescue, nimblewill, velvet grass, purple top, poison ivy, corn, oats, wheat, rye, buckwheat, peach, plum, pear		
Sunflower *(Helianthus scaberrimus)*	*Helianthus scaberrimus*			homologous in spring	22, 26

TABLE II (cont.)

PLANTS REPORTED TO PRODUCE DIFFERENTIAL GROWTH-INHIBITORS OF UNKNOWN COMPOSITION

Plant Sources	Plants Inhibited	Parts Inhibited	Plants Resistant	Remarks	References
Rye	grapes				25
Antennaria fallax	*Antennaria fallax*			homologous	26
Aster macrophyllus	*Aster macrophyllus*			homologous	26
Erigeron pulchellus	*Erigeron pulchellus*			homologous	26
Horse thistle	oats				28
Euphorbia	flax				28
Domestic ryegrass *(Lolium multiflorum)*	Kentucky bluegrass, Chewing's fescue				30
Redtop	Chewing's fescue				30
Butternut *(J. cinerea)*	shrubby cinquefoil *(Potentilla fruticosa)*				46
Tridax procumbens	other weeds				47
Zacate *(Leersia hexandra)*	rice				59
Black locust *(Robinia pseudoacacia)*	white pine, barley				60, 79
White ash	white pine				60
Oats, alfalfa avocado, barley, cocklebur, Sudan grass	tomato crown gall				71
Evergreen creosote bush	evergreen creosote seedlings			homologous	81

SITE OF ACTION

In most cases that have been studied, the inhibitors were shown to exert their effects not on the plant as a whole but on a particular part. For example, coumarin and parasorbic acid affect the growth mainly of roots (2, 23, 64).

Although extensive laboratory studies have not yet been done on the inhibitor from black walnut *(J. nigra)*, field observations suggest that it, too, has a specific site of action, at least on some plants. Tomatoes grown near black walnut develop a characteristic wilting of the leaves, but the roots and stem are unaffected (21).

TOXICITY

Although plant growth-inhibitors are often referred to as "toxic substances", they are usually not particularly toxic by pharmacologic or toxicologic standards. Indeed, one of the most striking characteristics of some of these inhibitors is the wide gap between the concentration which inhibits the growth of sensitive plants and the concentration which acually kills those plants. Sometimes, as in byakangelicin from *Thamnosma montana*, the lethal concentration is 60 times the growth-inhibiting concentration (12). On the other hand, in other growth-inhibitors the inhibiting concentration is close to the lethal concentration for the sensitive plant species. Such inhibitors might well be considered toxic.

HOMOLOGOUS AND HETEROLOGOUS INHIBITORS

Most of the inhibitors which have been studied are heterologous, that is, they exert their effects only on other species of plants. However, in some plants, e.g., guayule (14, 78), the inhibitor is homologous. It inhibits the growth of seedlings of the parent species. It may, of course, also inhibit other species. The homologous inhibitor presumably aids in the perpetuation of the species by preventing overgrowth of plants on inadequate soil, with subsequent death of the entire group. The presence of homologous inhibitors has been reported for guayule (14, 78) and evergreen creosote bush (81) which are desert plants. In addition, some field observations suggest that plants in other habitats may also secrete homologous inhibitors. Cooper and Stoesz (22) and Curtis and Cottam (26) have reported that the prairie sunflower *(Helianthus scaberrimus = H. rigidus)* grows in such a pattern as to

suggest that each plant inhibits the growth of others of its own species. This pattern, called "fairy ring," resembles the "fairy rings" of mushrooms, although the latter probably are produced by an entirely different mechanism. Several other plants are reported to grow in similar patterns, presumably due to the presence of inhibitors (26).

SPECTRUM OF ACTIVITY

Plant growth-inhibitors are apparently quite selective in terms of the species and sometimes even the variety of plants which are affected. Our knowledge of this aspect of their action is necessarily limited and must inevitably remain so, since a thorough investigation of the spectrum of a single inhibitor would involve testing its effects on each plant species. Despite this limitation, enough studies have been done to show that most inhibitors are highly specific in their action. For example, on guayule seedlings, transcinnamic acid will inhibit growth in low concentrations, while with 100 times that concentration, there is no evidence of inhibition of tomato (15).

Similarly, the plants which are inhibited by these chemicals also show a selectivity in terms of the inhibitors to which they are sensitive. For example, tomato, which is highly resistant to transcinnamic acid (14), is extremely sensitive to byakangelicin (12), isopimpinellin (12) and 3-acetyl-6 methoxybenzaldehyde (15, 38, 39) among other substances.

Unfortunately, comparatively few studies have been done on the spectra of purified inhibitors. Accordingly, it is necessary to fall back on some field observations for some of the most illuminating examples of inhibitors acting selectively on some species and not others. Perhaps the most extensively studied plant in this group is the black walnut *(Juglans nigra)*. It has been reported that this plant inhibits the growth of apple but not of pear, peach or plum trees; several common grasses but not Kentucky bluegrass or timothy; blackberry but not black raspberry, even though both belong to the same genus (18).

CHEMICAL CONSTITUTION

The growth-inhibitors which have been identified chemically do not show any similarity in structure (Table II). This parallels the situation with antibiotics. There are no important chemical similarities between many antibiotics, e.g., penicillin, achromycin, streptomycin and

chloramphenicol, even though there are several overlappings in their spectra of activity.

RELATIONSHIP OF GROWTH-INHIBITORS TO LIFE CYCLES IN THE PLANT

Extensive search has failed to reveal any reports of controlled studies to determine whether growth-inhibitors are elaborated throughout the life of a plant or whether they are secreted during certain periods only. However, some observations suggest that for some plants, the growth-inhibitors are more concentrated at certain times in the life cycle. MacDaniels and Muenscher, cited by Brooks (18), reported that black walnut seedlings up to two years old do not secrete a growth-inhibitor. Struckmeyer and Roberts (71) were able to extract a growth-inhibitor from the leaves of certain plants only during flowering.

In general, it has been known for centuries that certain potent medications derived from plants are found in high concentrations only during a limited part of the plant's life cycle. Therefore, it seems possible that differential growth-inhibitors in many cases are secreted in high concentrations only at specific times in the plant's life.

PERSISTENCE IN SOIL

There are wide variations in the degree of persistence of different inhibitors in the soil. Transcinnamic acid (15) is rapidly destroyed by bacteria, so that its effective life in the soil is only a few days.

On the other hand, Schreiner and his colleagues isolated from soil several inhibitors which apparently persist for years (66-69). Furthermore, it was reported by De Candolle (28) that certain plants such as wheat, could not grow well on land on which flax had once grown. This suggests long persistence of a growth-inhibitor from flax.

ECOLOGIC CONSIDERATIONS

The actions of growth-inhibitors may help explain several ecologic phenomena. At present there is no really adequate explanation for the single stand. Attempts to explain this phenomenon on the basis of superior adaptability to a particular habitat lose effectiveness when faced with the observation that in similar habitats there may be a single stand of another species or a wide mixture of plants. On the other hand, the single stand can be adequately explained on the basis that

some plants secrete a broad-spectrum inhibitor which is completely heterologous. Thus, when the density of these plants in a particular area passes a critical point, they will prevent all other plants from growing there, and they become a single stand.

Another phenomenon, the "fairy ring" seen in certain flowers, has been ascribed to differential growth-inhibitors (22, 26). In these cases, however, the inhibitors are presumably homologous, inhibiting plants of the same species. They might, in addition, include other plants in their spectra of activity, but this has not yet been clarified.

The composition of plant communities may also be determined in large part by the interaction of various growth-inhibitors as well as the usual environmental factors (80). This effect was suggested by De Candolle in 1828. He gave several examples of plants which inhibit the growth of other plants, and postulated that a growth-inhibitor exuded from the roots was the cause. The relation of black walnut to plant communities has been extensively investigated by Brooks (18). He found that in areas with similar soil and climatic conditions, the presence of black walnut trees is a dominant factor in determining the composition of the plant community. The plants growing within 50 feet of a black walnut form a decidedly different community than those growing around other kinds of trees in the immediate area. Since there were no significant variations in the soils in the two locations, the differential growth-inhibitor secreted by the black walnut was presumed to be the primary reason for the difference.

The ecologic patterns related to the growth-inhibitors may furnish valuable leads for further studies of these substances. For example, a botanist looking for more differential growth-inhibitors would probably have a greater chance of success if, instead of testing many plants selected at random, or on taxonomic grounds, he studied plants known to grow in a single stand, to form a fairy ring, or to exhibit consistent incompatibilities in plant communities.

AGRICULTURAL CONSIDERATIONS

The initial observations of De Candolle were stimulated by a desire to improve agricultural yields. He felt that in many cases, poor crop yields were caused by the presence of growth-inhibitors in the soil. He pointed out that French peasants had apparently been aware of this for generations before, giving as an example the cockscomb which affects grasses.

Schreiner et al. (66-69) also became interested in growth-inhibitors largely because of agricultural considerations.

There are a few concrete examples of the economic importance of growth-inhibitors. Schneiderhan, for example (65), has pointed out the economic loss resulting in Virginia from having black walnut trees near apple orchards.

Interactions between grasses in a mixture probably involve the effects of the differential inhibitors (1, 16, 30). In designing grass mixtures for various localities, it would presumably increase efficiency if the interactions were considered. For example, since Kentucky bluegrass inhibits Canada bluegrass, it seems that mixtures containing both bluegrasses would not grow as well as one containing either alone. Of course, the actual problem is more complex, since some grasses inhibit the Kentucky bluegrass.

Empirically, it has been known for centuries that weeds are injurious to crops. However, the mechanism of such injury has never been adequately explained. A common explanation has been that weeds take from the soil, moisture and nutrients needed for the cultivated plant. Careful scrutiny of such an explanation, however, reveals certain weaknesses. Most weeds are much smaller than the cultivated plants with which they compete. The weight of total corn plants per acre of cultivated ground is certainly higher than the total weight of weeds per acre, even where weeds are allowed to grow unchecked. If competition were solely on the basis of ability to utilize soil nutrients and moisture, one would expect that many food crops would outgrow the weeds. The argument might be raised that the weight of weeds is not a good criterion with which to measure uptake of moisture and nutrients; weeds might be very inefficient in their utilization of these materials. However, the ability of weeds to grow on marginal soils which will not support food crops suggests that most weeds are, if anything, more efficient in their utilization of moisture and nutrients. Thus it appears that some additional mechanism is involved in the competition between some weeds and crops. In a few cases it has already been suggested that this mechanism involves secretion of a differential growth-inhibitor by the weed (28, 76). Some unpublished experiments of the author suggest that young ragweed inhibits corn growth in a similar fashion.

It appears that an intensive study of weeds for growth-inhibitors might be of great economic benefit. Furthermore, in developing new

strains of useful plants, it might be worthwhile to look for resistance to growth-inhibitors as a potentially favorable genetic characteristic.

In 1902 Jones and Morse made the ingenious suggestion that butternut trees *(Juglans cinerea)* be planted extensively in order to eliminate shrubby cinquefoil *(Potentilla fruticosa)* which was a particularly difficult weed (46). Apparently, they were thinking in terms of differential growth inhibition. Their suggestion was not widely followed, but the basic principle may prove applicable to other areas where conventional methods of weed control are inapplicable.

COLCHICINE AND PODOPHYLLIN

Colchicine, derived from the meadow saffron *(Colchicum autumnale)*, and podophyllin from the May apple *(Podophyllum peltatum)* have certain similarities to differential growth-inhibitors but appear to differ in important respects.

Colchicine and podophyllin can produce polyploidy, but this effect has not been reported for any of the inhibitors. Furthermore, there are no reports of differential actions of either of these two agents. They apparently affect all multiplying cells (6, 19, 23, 29, 37, 49, 72).

On the basis of the available evidence, it thus appears that colchicine and podophyllin cannot be considered to be differential growth-inhibitors.

EFFECTS OF PLANT GROWTH-INHIBITORS ON ANIMAL CELLS

It is a matter of considerable interest that many plant growth-inhibitors will inhibit the growth and/or multiplication of animal cells. Those inhibitors which have a broad spectrum of activity for plants seem to have a similarly broad spectrum for animal cells. Furthermore, the patterns of activity are similar. Protoanemonin from *Anemone pulsatilla* (4) affects rapidly multiplying cells of plant or animal origin but not resting cells (31, 37, 77). It shows no particular specificity for any kind of rapidly multiplying animal cells, affecting fibroblasts and epithelial cells about equally (45). In plants it inhibits mitosis in prophase or interphase, but unfortunately search has failed to reveal reports of its exact action on animal cells.

Of growing interest are those inhibitors with a narrower spectrum of activity which affect certain kinds of cells more than others. The most

extensively studied thus far has been parasorbic acid. This material is found primarily in the ripe berries of mountain ash *(Sorbus aucuparius)* (55) but is also present in wheat "middlings," orange rind and perhaps other sites as well (54). It completely inhibits growth of tomato and onion roots (20, 23, 24). It has been reported and confirmed (44, 54, 63) that parasorbic acid inhibits animal cells of fibroblast origin but not of epithelial origin. It has subsequently been reported that this difference is quantitative, not qualitative, in that higher concentrations of parasorbic acid inhibit epithelial cells, too (42, 43). Nevertheless, it is clear that this material has a definite differential effect on animal cells (17).

RELATION TO CANCER RESEARCH

Recently, there has been growing interest in the possibility of finding a useful treatment for cancer among growth-inhibitors of plant origin. Since there has been a great deal of confusion in the medical literature about cancer growth, it may be worthwhile to digress slightly and to consider an aspect of cancer which may be related to the possibility of discovering in plants an agent to combat it. Cancer is widely regarded as a condition in which cells multiply without restraint and at very rapid rates. It will be useful to scrutinize the concept that cancer cells multiply rapidly. To be sure, this may seem the case to the unfortunate victim and his family. However, it is easy to show that several kinds of normal cells, essential to life, multiply far more rapidly than cancer cells. In the normal human the cells of the bone marrow multiply rapidly enough to produce about 2,500,000 red blood cells each second for a lifetime. After hemorrhage this rate can go up sevenfold. The cells lining the intestine multiply at least as rapidly, and there is some evidence to suggest that they may multiply even more rapidly than the bone marrow cells. Even the cells lining the mouth, which are not as important to life, multiply at faster rates than cancer cells. It is not an uncommon experience to scrape or cut the mouth lining and discover advanced healing within a few hours. Since normal cells, essential to life, multiply more rapidly than most cancer cells, it follows that a broad-spectrum inhibitor, whether synthetic or naturally occurring, which affects all rapidly multiplying cells will affect the bone marrow and intestinal lining at least as much as the cancer. The likelihood is that such a material might temporarily slow tumor growth, but before stopping it completely, would kill the patient.

On the other hand, narrow-spectrum inhibitors, which affect one kind of cell much more than others, offer considerable promise. Theoretically, it is possible to find among such inhibitors one which will affect a particular cancer more than normal cells. Such specificity of action would not be entirely unexpected among materials which affect one kind of bluegrass but not another, blackberries but not black raspberries, roots but not stems, leaves but not roots, etc. Indeed, there is considerable encouragement for such a view in reports on two of the differential inhibitors. Parasorbic acid (see above) apparently produces complete cures of the Jensen sarcoma in rats (44, 55). This is based on differential effects on various cells. Jensen sarcoma cells are extremely sensitive to the inhibitor, and it is thus possible to find a dose of parasorbic acid which completely inhibits tumor growth in the rat while not seriously affecting growth of cells essential to life.

Roberts has found that a plant growth-inhibitor obtained from leaves of oats, alfalfa, avocado, barley and other plants (71) is able to inhibit the growth of certain mouse tumors without killing the animal (personal communication). This material is currently under investigation.

Another plant growth-inhibitor, arbutin (66), has been reported to have effects in certain cancers (15C). In mice with the transplanted sarcoma 180, arbutin increased the life span.

There are also occasional, tantalizing notes scattered throughout the medical literature, reporting impressive therapeutic results from the use of plant extracts in human cancers (55A, 62A). Unfortunately, there is seldom, if ever, any indication whether the observation has been confirmed or not.

In the laboratories of the Albany Medical College, we are investigating the effects of plant growth-inhibitors on a wide variety of animal tumors. Others are studying the effects of plant extracts on cancer in a more general fashion. Apparently, they were not led to their present approach by considering the actions of plant growth-inhibitors but through another avenue. Most of the national testing program in cancer has involved screening of pure compounds made by organic chemists. However, even a cursory glance at a list of useful drugs reveals that in many areas, chemists cannot do as well as plants. For example, digitalis glycosides, used in heart failure, are found in many species of plants. Nevertheless, even though their structure is known, no chemist has ever succeeded in synthesizing an active glycoside. Accordingly, it is logical to suspect that plants may have anticancer materials which are

not synthesizable by chemists at our present state of development. Several investigators studying plant extracts on cancer have reported encouraging findings of damage to, or inhibition of, tumor cells (7–10, 32, 33, 41, 52, 53, 73-75). There is a growing number of plants whose extracts have been reported to either damage or inhibit tumor growth in pilot laboratory experiments. It is of interest to note that some, e.g., *Euphorbia* (7, 75) and *Trifolium* (9), are also listed in Table II as secreting plant growth-inhibitors. Furthermore, there are several cases in which one species of a genus, not tested for effects on cancer, is reported to secrete a growth-inhibitor, while a closely related species is reported to affect cancer growth; examples are *Anemone* (52) and *Prosopis* (52, 74). It would be of considerable interest to determine whether the proportion of differential growth-inhibitors among these plants affecting cancer in animals is higher than could be expected on the basis of chance alone.

There is reason to hope that one or more differential growth-inhibitors of plant origin may prove useful in human cancers. The available evidence suggests that if such materials are found, they will have relatively narrow spectra of activity, affecting one kind of cell much more than others. It is hoped that botanists will be on the look-out for such inhibitors and will cooperate in joint programs to test them on a wide variety of animal tumors.

SUMMARY

Differential plant growth-inhibitors appear to play a substantial role in many botanical relationships. They may prove to be important factors in agriculture and ecology. They are probably widespread throughout the plant kingdom and are found in various parts of plants. Their actions are usually specific, both in terms of the species and in terms of the part of the plant affected. Some inhibitors include individuals of the same species in their spectrum of activity. Although a few have been identified chemically, most have not.

There is a growing interest in the actions of plant growth-inhibitors on animal cells. In several cases it has been shown that materials can selectively inhibit multiplication of a particular kind of tissue in animals, including mammals. This may prove to be a crucial point in cancer research, and it is hoped that botanists who isolate such materials from plants will cooperate in making them available also for cancer research projects.

ADDENDUM

In a recent review article, Börner (15A) has considered many aspects of growth-inhibitors which are not covered in the foregoing. He has tabulated the liberation of amino acids and sugars from the roots of higher plants, and from seeds and fruits. The gaseous excretions, and germination-inhibitors were discussed. Börner also points out that certain microorganisms decompose non-toxic excretions of higher plants, producing phytotoxic substances. He concludes that in most species of plants, massive secretion of organic materials from intact uninjured roots probably does not occur. However, in certain cases, such secretion does occur, and particularly with crops, which are available in such large amounts, Börner concludes that "... there is enough evidence to be certain that some soils are made poisonous to plants by organic compounds from preceding crops."

In another article (15B), Börner describes an investigation into the excretion of phlorizin from apple root residues. He found that both the phlorizin and the first metabolic product, phloretin, inhibited the growth of apple seedlings in water culture, even at concentrations of 10^{-4}M, and with marked effects at 10^{-3}M. Whether or not these compounds are effective under natural conditions is not certain. Phlorizin would, on the basis of Börner's work, be classified as an homologous inhibitor.

Mergen (55A) has investigated a toxic principle found in the leaves of *Ailanthus altissima*. The material was not identified chemically. When tested on 46 species of plants, it adversely affected the growth of 45 but to varying degrees. The one resistant species was *Fraxinus americana*. Two aspects of Mergen's paper are of particular interest. First, he notes the natural growth of *Ailanthus* in pure stands, and suggests a causal relationship between the secretion of the toxic principle and the pure stand. Secondly, he points out that *Ailanthus* is also toxic to animals. Fourteen other hardwood species were tested, and only one, *Paulownia tomentosa*, appeared to have any toxic principle, and it was rather weak. The principle in *Ailanthus* appears to be a heterologous growth-inhibitor with a wide spectrum, but until it is further identified, it is not possible to rule out the presence of multiple inhibitors.

Muller and Muller (57) described association patterns in desert plants, and suggested that the complexion of plant communities is frequently the result of degrees of toxin production by some plants

and of toxin tolerance by others. They noted that in the natural habitat, the effectiveness of the growth-inhibitors may be markedly limited by such factors as microbial activity, adsorption to soil colloids or inactivation under conditions of dryness.

Nielsen et al. (58) studied the effects of several common crops on germination and on growth of seedlings of six species. Alfalfa extract was most potent in inhibiting other species, with timothy next. A marked difference in sensitivity to various extracts was noted in the species tested. The most sensitive was timothy, a species which, on the other hand, is quite insensitive to black walnut and clover (Table II). The importance of techniques in studies of this nature was stressed.

An extract of the periwinkle (*Vinca rosea*) has exhibited beneficial growth-inhibitory effects in certain human tumors, and is now in clinical use. The material, vincaleukoblastine, sold under the trade name Velban (Lilly), has the medical generic name of vinblastine sulfate. The inhibitory effects are striking on several types of mouse tumor (45A), on human choriocarcinoma (44A) and Hodgkin's disease. In addition, there is also inhibition of the normal bone marrow, so that a shortage of white cells occurs. This latter aspect of the inhibitory spectrum of vinblastine is undesirable, and constitutes a major limitation to its extended clinical use. It would be of great theoretical and practical interest to find out whether vinblastine is also a differential growth-inhibitor for plants, and to determine its spectrum of activity for plant species.

Additional aspects of this subject are considered in the article by Woods (82).

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